Volume 33, Number 1, August, 1960

The Yale Journal of Biology & Medicine

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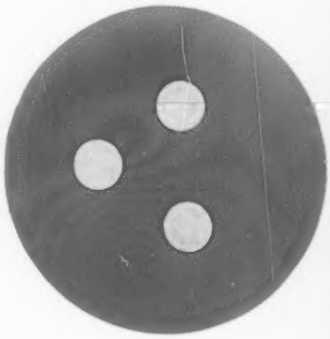

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Entered as second-class matter January 11, 1929, at the post office at New Haven, Connecticut, under the Act of March 3, 1879.

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PUBLISHED QUARTERLY FROM THE DEPARTMENT OF THE
HISTORY OF SCIENCE AND MEDICINE,
YALE UNIVERSITY, NEW HAVEN, CONNECTICUT

Distributor in Great Britain: Lloyd-Luke (Medical Books) Ltd.,
49 Newman Street, London, W.1.

TO ELIZABETH H. THOMSON

we dedicate this issue of the *Journal*, the first in a decade which has gone to press without the benefit of her editorial pencil.

We offer this as a token expression of our deep appreciation for the boundless time, energy, and skill which she has devoted to the volumes that have passed under her careful scrutiny. The vicissitudes of changing Editors, absent Editors, the problems inherent to special issues, the quiet training of the student editor—all have been met with understanding and insight, and the editorial continuity which she gave to the *Journal* has done much to maintain its standards.

It is with great regret that we accept Miss Thomson's resignation pressured by other editorial responsibilities within the University. It is with affectionate regard that we recognize her great contribution to the *Journal* and offer our wish for her continued success.

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W. LANE WILLIAMS*

Department of Anatomy, University of Mississippi School of Medicine, Jackson, and The University of Minnesota, Minneapolis

HEPATIC LIPOSIS AND MYOCARDIAL DAMAGE IN MICE FED CHOLINE-DEFICIENT OR CHOLINE-SUPPLEMENTED DIETS**

The feeding of hypolipotrophic diets produces rapid and extensive hepatic liposis in several species including rats[4] and mice.[2, 14] In addition to being deficient in choline the diets used in such experiments usually contain a large amount of fat and a marginal amount of protein.[10, 14, 18] Hypertension and renal glomerular lesions have been observed in rats fed such diets.[1] These observations have stimulated study of the actions of this type of nutritional deficiency in the production of lesions of arteries and myocardium.[5, 7, 23] There are contradictions related to the specific actions of the choline deficiency in several aspects of experimentally produced cardiovascular disease including injury to the heart and blood vessels,[17, 20, 21, 30] and plasma levels of lipids and lipoproteins.[20, 24]

In the present study major attention has been directed to hepatic liposis and to myocardial necrosis and fibrosis in young adult mice. The mice were older than the rats commonly used in such experiments. The selection of older animals was intentional so that a postweaning phase (four to six weeks) of adequate nutrition and normal growth could be established.

MATERIALS AND METHODS

Animals. Mice of the C (Bagg albino) stock with an initial body weight of 20-22 gms. were restricted to either the choline-deficient or choline-supplemented diet when 8 to 10 weeks of age.

Diets. From weaning (30 days of age) until eight to ten weeks of age these mice had been fed a standard laboratory ration that was adequate in choline, vitamins and minerals, being composed of approximately 25 per cent protein, 6 per cent fat, and 47 per cent carbohydrate. The composition of the choline-deficient diet was as follows:

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* Professor and Chairman, Department of Anatomy, University of Mississippi School of Medicine, Jackson.

** This investigation has been supported by grants from the National Institutes of Health, U.S.P.H.S. (H-728 and H-4052; A-0648 and A-2693 M. & N.) and from the Minnesota Heart Association and the Mississippi Heart Association.

Received for publication 30 November 1959.

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	Gms.
Vitamin-free casein	80.0
Sucrose	480.5
Lard	400.0
Salt Mixture (No. 2, U.S.P. XIII)	40.0
l-cystine	5.0
Cod liver oil	4.5
Vitamin powder	10.0

The vitamin powder contains:

Thiamine hydrochloride	0.500
Riboflavin	0.250
Pyridoxine hydrochloride	0.200
Calcium pantothenate	1.000
Nicotinic acid	1.000
Powdered sugar	997.050

The choline-supplemented diet contained 5 gms. of choline chloride in 1000 gms. of the diet described above.

Within the mouse cages the food was kept in metal containers constructed with partitions and wire-mesh covers to restrict contact with the diet to the mouth and snout. A wire-mesh floor was placed above the permanent floor of the cage to limit coprophagia.

Mice were weighed daily during the first month of feeding the experimental diets and weekly thereafter.

Histological technics. Abdominal and thoracic organs were fixed in 10 per cent formalin or in Lavdowsky's solution[20] or in both. Hearts were cut into two relatively equal frontal sections for fixation in 10 per cent formalin and Lavdowsky's. Thigh muscle from about one-half of the mice, and diaphragm from essentially all were fixed in Lavdowsky's solution. Routine paraffin sections (10 micra) of all of these organs and tissues were stained with hematoxylin and eosin (H. and E.).

Fat was demonstrated by staining frozen sections of formalin fixed material with oil red 0 in the same manner as with other Sudan dyes.[14, 17] It was essential to use gelatin embedding[18] to make adequate frozen sections of the hearts. As the result of technical errors only 25 per cent of these specimens were stained satisfactorily for fat.

The PAS (periodic acid-Schiff's reagent) technic was used to show glycogen and ceroid pigment. Ceroid pigment was also demonstrated by staining dehydrated and defatted (i.e., embedded in paraffin) specimens with dyes such as oil red 0 and Sudan black. The Masson (with aniline blue), the PAS and the Laidlaw reticulum methods were used to show fibrosis, and the von Kossa technic to demonstrate minerals. The staining and histochemical procedures mentioned above were used as described by Lillie.[18] The specific uses of material prepared by these histological technics are described later.

Selection of mice for study. At the intervals shown in Table 3, mice were killed by digital compression of the cervical spinal column. The usual procedure was to select those showing weakness, decreased activity, and respiratory difficulties plus extreme weight loss, provided the other signs of ill health just mentioned were evident. During the first 8 weeks of the experiment, such evidence of ill health was infrequent and

mice were killed on a schedule that produced a chronologic survey of changes in the livers of the members of the two dietary groups. Approximately 80 per cent of the mice killed after 8 weeks of feeding were females. Of those killed earlier, one-half were males. The males were extremely difficult to use in the experiments. They were active and expert in upsetting and burrowing into the food containers and also in becoming entrapped during such activities. In addition it was very time consuming to examine and weigh them since they objected very actively to handling.

Study of the material: Livers. One large lobe was fixed in Lavdowsky's solution (for subsequent paraffin-embedding) and another in 10 per cent formalin. Six to eight sections of the latter (across the entire lobe) were stained to show fat. Complete sections of the paraffin embedded specimens were processed as follows: 6 to 8, H. and E.; 4 to 6, PAS; and when so indicated by the PAS, and/or H. and E., 4 to 6 additional sections were stained by the Laidlaw reticulum method. Ceroid pigment is recognizable in H. and E. and in PAS preparations and also in frozen sections stained with Sudan dyes. When the pigment was so observed more sections (paraffin) were cut and then stained with Sudan dyes.

Hearts. One-half of each heart (complete frontal or saggital section as in Figure 13) was embedded in paraffin and the other was kept in formalin (10 per cent) for frozen sections and also as reserve material. At least 80 per cent of every heart and all of a majority were completely consumed in making the following preparations, all of which had some value in identifying areas of myocardial fibrosis and other lesions: H. and E., Masson, PAS, Laidlaw reticulum (in a variety of combinations with PAS and H. and E.), von Kossa, and frozen sections. Subsequent to frozen sectioning the remaining portion of the formalin-fixed half of the heart was dehydrated and embedded in paraffin and prepared for study by the staining procedures mentioned above. The numerous sections of the heart gave ample opportunity for studying structures located at or near the base, including aorta, vena cava, pulmonary vessels, thymus, lymph nodes, nerves, autonomic ganglia, unilocular and multilocular fat, esophagus, bronchi, and a large number and variety of small blood vessels within or near these several structures.

Other organs and tissues. Adrenals and kidneys as a single "block" were cut into two equal portions in the usual fashion, one being fixed in Lavdowsky, the other in 10 per cent formalin. Two to four sections were stained with H. and E. and a similar number with PAS. Two to four frozen sections were stained with oil red O. Skeletal muscle (diaphragm, thigh and esophagus) and gonads and spleen were studied in two to four sections stained with H. and E. In most instances two to four sections of adrenals (embedded in paraffin and included in same section as kidney) and gonads were stained with Sudan black to demonstrate ceroid pigment.

The simple statistical analysis (means and standard deviations) of the data on body weights (Tables 1 and 3) was based on the methods and tables presented by Croxton.[8]

OBSERVATIONS

At autopsy the significant findings in mice killed after more than eight weeks of restriction to the diet were depletion of the usual fat depots, pneumonia, and fatty livers (in the choline-deficient group). Other tissues and organs, including the alimentary tract, appeared normal.

TABLE 1. WEIGHT CHANGES IN MICE FED HIGH FAT(LARD)-LOW PROTEIN DIETS

	Choline-deficient diet:				*Choline-supplemented:*			
Months fed	*Total no. of mice*	*Mean weight change*	*Per cent of mice: Gained weight*	*Lost weight*	*Total no. of mice*	*Mean weight change*	*Per cent of mice: Gained weight*	*Lost weight*
		%				%		
1	84	—1.2 ±6.5	41 (6.1) ±2.9	47 (7.7) ±4.6	47	+0.86 ±8.8	55 (6.8) ±8	38 (8.4) ±7.9
2	69	—4.2 ±9.8	29 (10) ±7.3	60 (12) ±7.8	37	—0.8 ±12.8	59 (8.4) ±4.5	41 (14.3) ±10.1
3	31	—1.0 ±11.1	45 (9.5) ±6	55 (10.2) ±7.5	34	—6.6 ±12.9	41 (5.7) ±6.2	50 (20) ±11
4	23	—3.9 ±14.8	52 (9.8) ±7.2	48 (18.7) ±10.3	28	—8.6 ±9.2	28 (3) ±3.3	64 (15) ±8.4
5	18	—4.3 ±11.9	44 (8.3) ±5.2	56 (14.4) ±10.8	24	—15.3 ±10.1	8 (2.5)	92 (16.9) ±9.6
6	15	—5.0 ±9.4	33.3 (7.7) ±2.4	66.6 (11.5) ±9.1	9	—12.4 ±7.2	0	100
7	12	—10.4 ±7.9	8.5 (3.2)	83 (13) ±8.3	8	—17.0 ±8	0	100
8	9	—11.7 ±13.1	0	100	2	—20.2	0	100
9	8	—20.0 ±8.4	0	100	No survivors			
10	6	—24.2 ±4.1	0	100	No survivors			

(a) Weights are those of all mice living at the stated intervals.

(b) Numbers in parentheses are means of weight changes (%) in animals (% of total in group) listed directly above.

(c) Standard deviations are those of changes in body weight (i.e., the mean listed directly above) and were not determined for groups smaller than 5 mice.

Livers. The results are summarized in Tables 2 and 4. In chronologic order the livers of mice fed the choline-deficient diet showed: (i) complete lobular liposis within 72 hours; (ii) formation of fatty cysts within 5 weeks; (iii) ceroid pigment within 9 weeks; and (iv) within 22 weeks a nodular hyperplasia of the parenchyma and an apparent increase of reticular fibers between these nodules. Some of these changes are shown in Figures 1, 2, and 5, and all have been described in detail previously.[14] The term fatty cyst as used here refers to structures previously described.[2, 9, 14]

TABLE 2. CHANGES IN LIVERS AND HEARTS OF MICE FED HIGH FAT-LOW PROTEIN DIETS

Livers:	*Choline deficient (% of mice)*	*Choline supplemented (% of mice)*
	12-40 wks.	*12-33 wks.*
Liposis Absent	0	45%
Complete Lobular Liposis	100%	0
Fatty cysts	100%	0
Ceroid pigment	100%	0
	27-40 wks.	*27-33 wks.*
Reticulinosis and Parenchymal Hyperplasia	100%	0
Myocardial lesions:		
1- 8 weeks:	0	0
9-15 weeks:	0	50%**
16-33 weeks:	25%*	72%**
34-40 weeks:	31%*	No survivors

* Small areas of fibrosis.
** Areas of extensive fibrosis.

No fat (sudanophilia in frozen sections) was observed in the livers of approximately one-half of the mice fed the choline-supplemented diet for the entire period of 1 day to 33 weeks (Tables 2 and 4). In an approximately equal number of mice the hepatic liposis was evident but was limited to very small sudanophilic droplets which did not fill or distort parenchymal cytoplasm or involve all zones of lobules (Figs. 3, 4, 6). Livers of mice fed this diet for as long as 33 weeks and who had lost as much as 25

per cent of body weight appeared normal when stained with hematoxylin and eosin or by the PAS (plus hematoxylin) method for demonstrating glycogen (Figs. 4, 6).

Hearts. There was myocardial injury in both of the dietary groups. A precocity, higher incidence and greater extent of myocardial lesions were

TABLE 3. MYOCARDIAL LESIONS

	Choline-deficient diet:				*Choline-supplemented diet:*			
Weeks fed diet	*No. of mice killed*	*Per cent with lesions*	*Weight loss: (per cent) with lesions*	*Weight loss: (per cent) without lesions*	*No. of mice killed*	*Per cent with lesions*	*Weight loss: (per cent) with lesions*	*Weight loss: (per cent) without lesions*
1- 8	84	0	—	4.2* ±9.2	30	0	—	0.8* ±11.1
9-15	7	0	—	33 ±5.7	10	50	30 ±9.8	34 ±10.1
16-21	23	30	35 ±4.1	34 ±7.9	15	66.6	17 ±10.1	26 ±3.9
22-27	21	24	16 ±8.1	19 ±9.1	9	56	17 ±9.3	26 ±3.9
28-33	8	12.5	18 **	16 ±8.1	8	100	24 ±9.7	—
34-39	7	28.6	22 **	20 ±7.7	No survivors			
40	6	33	26 **	23 ±4.8	No survivors			
	9-40 wks. 72 mice	23.6%			9-33 wks. 42 mice	66.6%		

* Av. wt. changes in all mice (killed and surviving) at 8 weeks.
** No standard deviation determined for groups smaller than 4 mice.

obvious in the choline-supplemented group (Tables 2 and 3). A significant incidence of myocardial lesions (50 per cent) occurred in choline-supplemented mice killed during the 9-15 week interval of feeding this diet. This incidence was greater than in mice fed the choline-deficient diet for 16-40 weeks (Tables 2 and 3).

The most frequent cardiac lesion was fibrosis of the ventricular myocardium. In mice fed the choline-deficient diet these lesions were in-

TABLE 4. CHANGES IN LIVERS OF CHOLINE-DEFICIENT AND OF CHOLINE-SUPPLEMENTED MICE

Weeks on diet	*No. of mice*		*Per cent of mice showing*											
			Liposis absent		*Incomplete lobular liposis*		*Complete lobular liposis*		*Fatty cysts*		*Ceroid pigment*		*Reticulinosis and parenchymal hyperplasia*	
	−C	*+C*	*−C*	*+C*	*−C*	*+C*	*−C*	*+C*	*−C*	*+C*	*−C*	*+C*	*−C*	*+C*
1-4	42	15	0	40	0	60	100	0	0	0	0	0	0	0
5-8	42	15	0	60	0	40	100	0	100	0	0	0	0	0
9-15	7	10	0	70	0	30	100	0	100	0	14	0	0	0
16-21	23	15	0	60	0	40	100	0	100	0	100	0	0	0
22-27	21	9	0	11	0	89	100*	0	100	0	100	0	33	0
28-33	8	8	0	37.5	0	62.5	100	0	100	0	100	0	100	0
34-39	7	0	0				100		100		100		100	
40	6	0					100		100		100		100	

−C = choline-deficient diet. +C = choline-supplemented diet.

* Lobular pattern of parenchyma was greatly distorted after 24 weeks.

frequent (Tables 2 and 3), limited in size, and consisted of a restricted fibrosis. Lesions such as in Figure 7 appear to be early fibrosis in which fibroblasts and macrophages were numerous. Lesions of approximately the same size, but consisting chiefly of collagenous fibers were more characteristic of the hearts of choline-deficient mice. In this group of mice no lesions larger than the one shown in Figure 7 were seen.

In the hearts of choline-supplemented mice (Tables 2 and 3) the areas of fibrosis (Figs. 9-12, 15, and 16) were larger and multiple (Fig. 13). The fibrosis seemed to be a reparative process that was not encroaching upon myocardial components. Minimal and apparently initial fibrosis consisted of a small amount of ground substance and a large number of fibroblasts. In a more advanced stage of fibrosis the amount of interstitial material had increased and reticular fibers were present. A relatively definitive and older fibrosis was represented by a predominance of collageous fibers within the area. The areas of fibrosis usually contained what appeared to be fragments or remnants of myocardial fibers whose sarcoplasm was hypochromic when stained by eosin or by the acid fuchsin of the Masson technic (Figs. 9, 10, 11).

Active necrosis was not indicated by striking changes in myocardial fibers. In a few instances atrophy or necrosis (Figs. 9, 10, 11) was suggested by shrinkage of fibers, decreased eosinophilia, and poor definition of fibrillar structure. In one heart most fibers within the lesion were heavily stained with hematoxylin (Fig. 11). Ceroid pigment and mineral could not be demonstrated in such fibers and are assumed not to be responsible for the basophilia. Fibers that appeared hypochromic to eosin or acid fuchsin (Masson) also lacked the fine argyrophilic granulation of sarcoplasm that characterized normal myocardium stained by the Laidlaw method for reticulum (Fig. 14). Evidence of active myocardial necrosis was absent from hearts of all mice fed the choline-deficient diet and as described above was not clearly indicated in the cardiac lesions in mice receiving the choline-supplemented diet. Cellular reactions within the lesions were limited to an abundance of fibroblasts and to a small number of macrophages and lymphocytes. The number of neutrophils was not significant. Pericarditis (epicarditis) in which neutrophils and lymphocytes were abundant was present in approximately 10 per cent of the hearts with myocardial lesions. The pericarditis was similar to that described earlier in mice injected with large amounts of cortisone.[28] Endocardium, valves, and blood vessels of all layers of the hearts were normal. In the small number of satisfactory frozen sections of hearts stained with a Sudan dye there was no sudanophilia in walls of vessels or in the layers of the heart except the epicardium.

Vacuolation that might indicate liposis was lacking in the myocardium of the routinely defatted sections of heart.

Other organs. In sections stained with hematoxylin and eosin there were no significant changes in kidneys (also stained for fat), abdominal aorta, adrenals, or gonads. In adrenals and gonads the amount of ceroid pigment was usual for the age of the mice and apparently had not been influenced by the choline content of the diet. The blood vessels of these several organs were normal. Pneumonia was common in all mice fed the diets for more than two months. Mucosal inflammation was frequent in trachea and extrapulmonary bronchi. The great vessels at the base of the heart were normal. Multilocular fat (periaortic and perirenal) was unchanged. In mice fed the diets for three months or more unilocular fat was depleted in that cytoplasm was shrunken; nerves and autonomic ganglia appeared normal in H. and E. sections. The esophagus, including its smooth and striated musculature, showed no abnormalities. Lymph nodes, spleen and thymus showed no obvious depletion of mature forms or other alterations. Skeletal muscle (thigh, esophagus and diaphragm) was free of any changes including the deposition of ceroid pigment and calcium.

Relation of general health and of weight loss to changes in livers and hearts. After 8 weeks weight loss was greater in mice fed the choline-supplemented diet (Table 1). At all intervals the range in weight loss was broad in both dietary groups (Tables 1 and 3). After the first eight weeks, all mice killed for study represented the less healthy members of the dietary groups and the majority had lost considerable amounts of weight (Tables 1 and 3). The changes in the livers showed no relation to amount of loss in body weight. Very fatty livers were present in all of the choline-deficient mice and the other hepatic manifestations of the deficiency ensued in the expected relation to its duration (Tables 2 and 4; Figs. 1, 2, 5). Hepatic changes were not significant in the choline-supplemented mice (Tables 2 and 4; Figs. 3, 4, 5). The extent of liposis in the livers of members of this group seemed unrelated to changes in body weight.

Here it is not possible to present the complete history of weight changes of individual mice. There was no instance of rapid weight loss followed by significant gain of weight. Most commonly the rapid loss of weight took place during the two weeks immediately preceding the time at which the animal was killed.

A great majority of the heart lesions were in mice with extreme decreases in body weight. However, the weight losses of animals killed within a specific chronologic interval were not significantly or consistently greater in

those with cardiac lesions (Table 3). In mice killed during the 9-27 week-interval the incidence of myocardial lesions was more than 50 per cent greater in the choline-supplemented group. Extensive myocardial fibrosis was observed in five mice lacking excessive weight loss as follows: at 20 weeks, 4.5 and 5.3 per cent weight loss; 24 weeks, 1.8 and 4.2 per cent; and at 31 weeks, 9.8 per cent. In choline-deficient mice cardiac lesions were limited to mice with weight loss of more than 10 per cent at the time of death.

The constant finding of pneumonia in mice killed after eight weeks on the diets was mentioned earlier. Several experimental procedures seem to precipitate this disease in mice.[23, 24] It seems highly probable that pneumonia contributed significantly to the poor health including the weight loss observed in these animals.

DISCUSSION

The livers of mice fed the choline-supplemented diet appeared normal except for a very restricted and irregular liposis. The degree of protection that choline supplementation afforded to the liver was impressive. This same group of mice showed a precocity and an increased incidence of myocardial injury.

The frequent occurrence of myocardial lesions and mural liposis in aortas and coronary arteries of young rats fed high fat, low protein, choline-deficient diets has been responsible for the belief that an adequate intake of choline is essential for maintenance of the cardiovascular system in this species.[20, 21] Significant and perhaps contributory renal lesions occur very early in young rats fed similar diets.[1, 21, 22] The following were regular features of these earlier studies:[5, 6, 21] (i) The rats were young, often immediately post-weaning. (ii) Such diets produced a rapid and extensive hepatic liposis which was prevented by choline supplementation. Also, a considerable variety and number of dietary lipids were used.[20, 21] More recent observations in the rat,[10, 12, 17, 20] monkey,[13] and chicken[15] indicate that these earlier studies overemphasized the specific actions of the choline deficiency in the production of cardiovascular injury. In the present study the animals were of a different species and were older than the rats commonly used in studies of the actions of choline-deficient diets on the cardiovascular system, liver and kidneys.[1, 21, 22] Also in some of the studies on rats a majority of the animals were males[21, 22] rather than females as used here. There is evidence that testosterone aggravates actions of a high fat—low protein, choline-deficient diet on the cardiovascular system and the kidneys.[19]

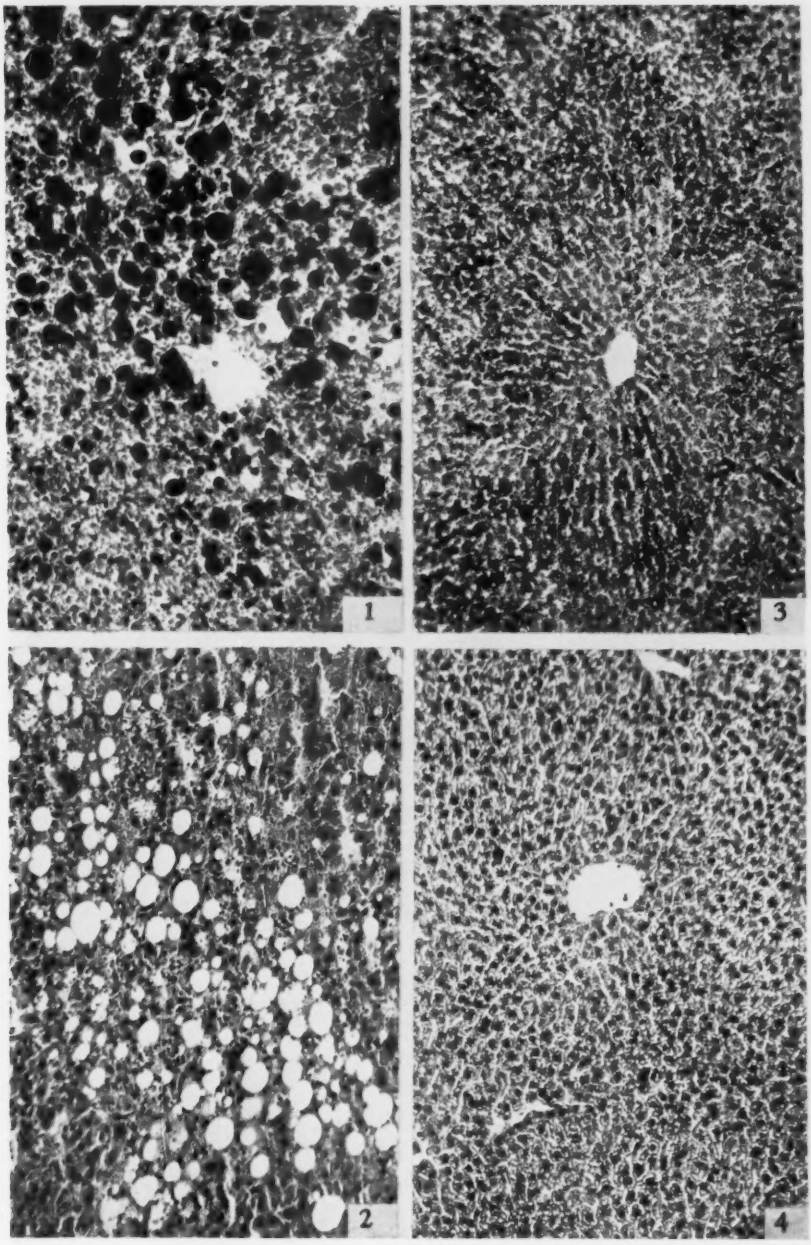


PLATE 1

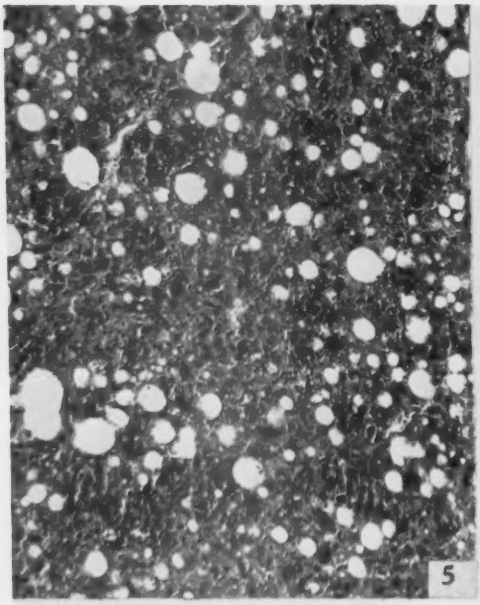
Livers (x100) of mice fed choline-deficient (Figs. 1 and 2) or choline-supplemented diets (Figs. 3 and 4) for 5 months.

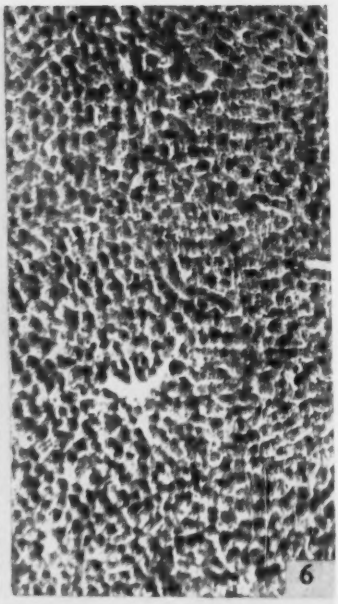
FIG. 1. Frozen section stained with oil red O and hematoxylin. All parenchymal cells contain fat. The large fat-filled structures (cysts) seem to be the result of rupture and fusion of adjacent cells.

FIG. 2. Same liver as in Figure 1. Paraffin section, H. and E. stain. Hyperplasia (apparently the first stage in formation of regenerative or hyperplastic nodules) is indicated by the large cells with hyperchromatic nuclei.

FIG. 3. The restricted and predominantly peripheral (portal) lobular liposis shown here represents the maximal amount seen in livers of choline-supplemented mice (frozen section, oil red O and hematoxylin).

FIG. 4. Same liver as in Figure 3, paraffin section, H. and E.





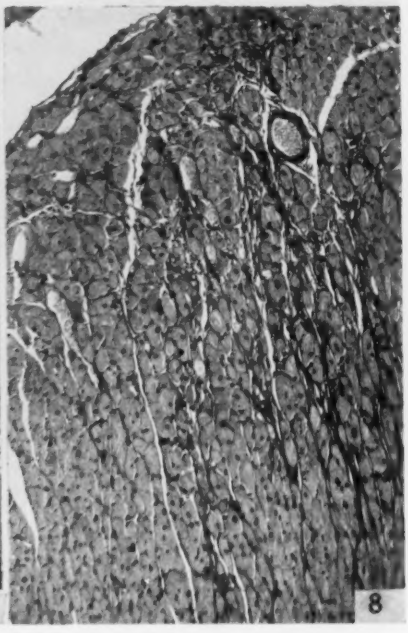


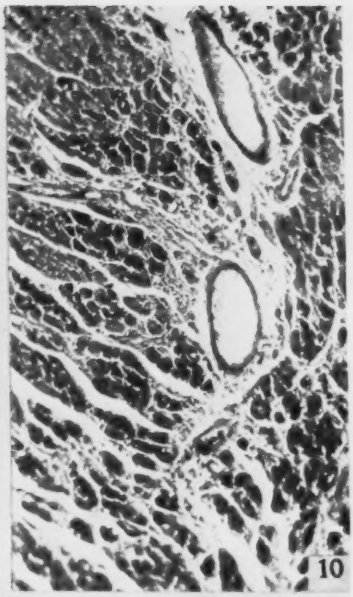
PLATE 2

FIG. 5. Liver of mouse fed choline-deficient diet for five months. PAS and hematoxylin. The darkly stained material is ceroid pigment. The cytoplasmic PAS-positivity indicating glycogen is very faint, x100.

FIG. 6. Liver of mouse fed choline-supplemented diet for five months. There is no staining reaction for ceroid pigment. Staining positivity indicating cytoplasmic glycogen is within normal range. PAS and hematoxylin, x100.

FIG. 7. Maximal (as to extent) type of myocardial lesion seen in choline-deficient mice, H. and E., x180.

FIG. 8. Restricted amount of myocardial fibrosis in choline-supplemented mouse, PAS, x115.



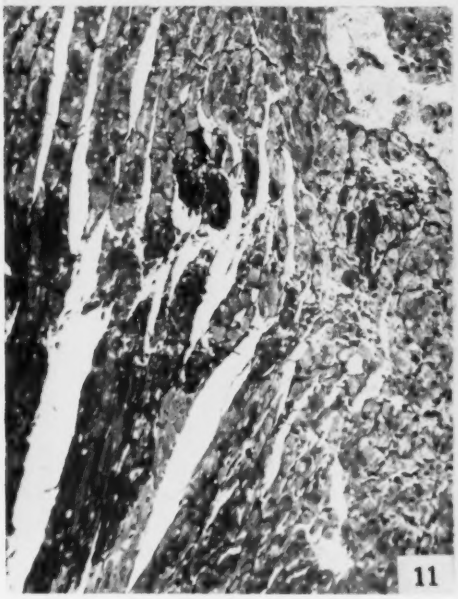


PLATE 3

Hearts of mice fed choline-supplemented diets for four and five months.

FIGS. 9 and 10. Myocardial fibrosis representing the most frequent lesion seen in hearts of choline-supplemented mice, (Masson stain). Figure 9, x115; Figure 10, x90.

FIG. 11. Myocardial necrosis and small areas of early fibrosis (Laidlaw reticulum plus H. and E.). Calcium and ceroid pigment could not be demonstrated in the very basophilic myocardial fibers, x115.

FIG. 12. Myocardial fibrosis, PAS, hematoxylin, x115.

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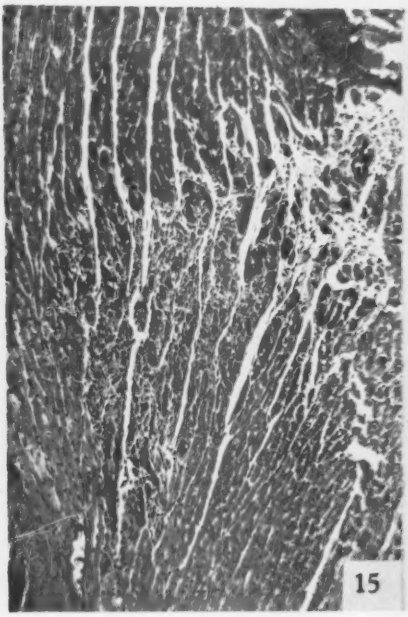

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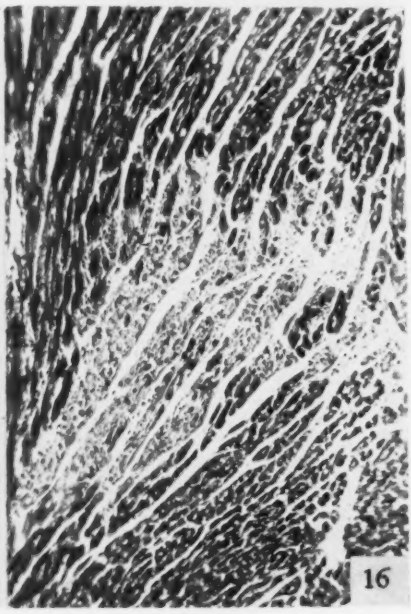

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PLATE 4

Hearts of mice fed choline-supplemented diets for four to five months.

FIG. 13. The rectangle encloses a lesion (or lesions) occupying a large area. The lesions, mostly fibrosis, are similar to those shown in Figs. 15 and 16 (below), x14.

FIG. 14. Argyrophilic granulation of sarcoplasm is lacking, but reticular fibers are stained in the several myocardial lesions shown here. Laidlaw method for reticulum, no counterstain, x140.

FIGS. 15 (H. and E.) and 16 (Masson) show large areas of myocardial necrosis and early fibrosis, x90.

The high incidence of cardiac lesions observed here might suggest that correction of the choline deficiency (with reference to hepatic liposis) produces a nutritional state conducive to the previously demonstrated myocardial necrogenic actions of low protein diets in mice.[2, 26] However, the choline-supplemented mice failed to show two common features of protein deficiency in this species. These are hepatic necrosis, and deposition of ceroid pigment and calcium in skeletal and myocardial muscle[2, 26] Dietary components acting separately and collectively in the production of injury to the liver and to the myocardium of mice and/or rats include the quantity and type of fat,[6, 11, 16, 27] amount of protein,[6, 26] levels of choline[21, 22, 30] and vitamin E,[26] and content of cystine[26] in relation to that of protein.

When results in rats[10, 17, 20, 21] and mice[2, 6, 26] are compared, the cardiovascular system seems to show considerable diversity in its reactions to atypical diets. In relation to a high fat diet neither species is as susceptible as the rabbit to production of atheromatous lesions.[15] In most of the studies mentioned earlier dietary lipid has consisted of lard, butter, or oil furnishing a wide range as to the chemical nature (degree of saturation and chain length) of the fats. Several studies indicate that the chemical nature of the dietary fat is important in relation to the amount and pattern of hepatic liposis[16, 27] and to the extent of injury to the cardiovascular system.[5, 6, 21, 23] Ethyl-laurate displays a great capacity to produce myocardial damage in young rats fed choline-deficient diets.[11] Buckley and Hartroft observed fat in coronary arteries and myocardium of choline-deficient mice, but questioned that the incidence was significantly greater than in their choline-supplemented group of mice.[2]

The following observations which seem significant are from studies[20, 21] of very young rats fed 12-60 days on high fat diets with protein contents of 15-30 per cent: (i) food intake was low in the several dietary groups; (ii) the incidence of myocardial liposis and necrosis seemed dependent upon the presence of a choline deficiency and to some degree the type of dietary fat; (iii) the incidence of such lesions was extremely low in the choline-supplemented rats; (iv) myocardial lesions were produced by feeding fat-free, choline-deficient diets. The incidence of myocardial damage is high in young rats fed high fat (40 per cent lard or butter plus 5 per cent cholesterol) diets containing propylthiouracil and sodium cholate with and without choline supplementation.[17] Supplementation with choline seemed to enhance the development of lesions described as coronary thrombosis and myocardial infarction.[17] The results of the present study indicate that a dietary level of choline sufficient to prevent hepatic liposis increases the susceptibility of the myocardium to high fat, low protein diets.

Within a given period the great range in weight changes of mice, both with and without myocardial lesions, precluded valid analysis of the relation of weight loss to the incidence of cardiac damage (Tables 1 and 3). The data permit no evaluation of the relation of food intake to weight loss. Because of the marginal dietary content of protein a true deficiency would exist when intake was low. As mentioned earlier the amount of weight loss of mice killed at the specific intervals could not be correlated with the presence or absence of myocardial lesions (Table 3). Objective consideration of the situation demands a broader view. In the choline-supplemented mice the early occurrence and relative rapidity of weight loss are obvious when the chronology and extent of changes in weight are considered for all of the mice (Table 1). The condition or conditions responsible for the early and rapid loss of body weight may have contributed significantly to the high incidence of myocardial lesions in the choline-supplemented mice.

The apparently healthy state of livers of choline-supplemented mice deserves attention in relation to the possible contribution of decreased food intake to weight loss and cardiac damage. In choline-supplemented mice with extreme losses in body weight the decreases in glycogen and increases in fat within hepatic parenchymal cytoplasm were less than are produced by relatively short as well as longer periods of fasting.[9, 23, 28] These livers also lacked the extreme changes including necrosis, cirrhosis and in some instances deposition of calcium that have been described in mice fed diets containing marginal amounts of protein, but with low fat content.[9, 28] The true actions of inanition could be determined by precise and extended studies of food intake with reference to essential requirements provided by the dietary protein, fat and carbohydrate in relation to several levels of choline-supplementation. The separate and joint actions of at least four dietary components must be evaluated. These are the choline-deficiency, the high fat content, the adequacy of the choline supplementation in relation to organs other than the liver, and the deficient (or at least marginal) level of protein.

SUMMARY

1. In chronological order the livers of mice fed a high fat, low protein, choline-deficient diet showed complete lobular liposis, formation of fatty cysts and ceroid pigment, nodular parenchymal hyperplasia, and distortion of the stromal pattern.

2. Supplementation of the diet with choline chloride prevented all of these hepatic changes except a very limited amount of parenchymal liposis.

3. In mice fed the high fat, low protein, choline-deficient diet for 9-40 weeks there was an incidence of 23.6 per cent of myocardial lesions consisting of small areas of fibrosis. In mice fed the choline-supplemented diet for 9-33 weeks 66.6 per cent showed myocardial lesion. Fibrosis was the predominant feature of a majority of these lesions. No significant changes were observed in kidneys or blood vessels of either dietary group.

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RICHARD J. SCHAIN*

Departments of Pharmacology and Psychiatry, Yale University School of Medicine

NEUROHUMORS AND OTHER PHARMACOLOGICALLY ACTIVE SUBSTANCES IN CEREBROSPINAL FLUID: A REVIEW OF THE LITERATURE**

There has been a considerable resurgence of interest in recent years in analysis of cerebrospinal fluid in neurological and psychiatric disorders. Four monographs and a review article have summarized much of the pertinent literature.[32, 45, 60, 88] The purpose of this review is to call attention to an area that has not been adequately described in the publications referred to above. It will comprehensively summarize the literature since 1910 dealing with neurohumors and other pharmacologically active substances in cerebrospinal fluid. An attempt will be made to organize in a critical manner some of this material, and to discuss further avenues of investigation.

INTRODUCTION

Neurohumors may be defined as highly active, naturally-occurring substances involved in neuronal activity, that are found in the nervous system in such minute concentrations, as to generally require bioassay techniques for their determination. (Spectrophotofluorimetric methods have also been used for this purpose in recent years). Substances usually considered as neurohumors are listed as follows:

1. Acetylcholine—a quarternary ammonium compound
2. Biogenic amines—
   a. catecholamines
      (i) noradrenaline
      (ii) adrenaline
   b. 5-Hydroxytryptamine (serotonin)
   c. Histamine

Substance P (a polypeptide) and dopamine (a catecholamine) have also been considered as potential neurohumors, but have never been reported in cerebrospinal fluid. The same is true of gamma-aminobutyric acid (GABA), although the peculiarly high concentrations of this substance found in brain would make GABA an exception to the rule that neurohumors are found in minute amounts in nervous tissue.

* USPHS Trainee, Section of Neurology, Yale University School of Medicine. Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7, England.

** This investigation was supported by Special Traineeship NBI-EP BT-507 from the National Institute of Neurological Diseases and Blindness.

Received for publication 1 March 1960.

Giarman[32] and Crossland[17] have recently published reviews of current concepts of the neurophysiological significance of these substances found in brain. Briefly, it may be stated that neurohumors are thought to play a role in transmission of the nerve impulse, both in the peripheral and central nervous systems. This role may be that of direct synaptic transmission of the impulse to the subsynaptic membrane, as is probable with acetylcholine, or it may be that of a 'neuroregulatory' agent modulating neural function in some, as yet unknown, manner. This latter concept has been developed as a result of the accumulation of a large body of data on amines that refuses to fall into any simple scheme and yet makes it apparent that many of these substances are intimately involved in neuronal activity.

The problem of interpretation of data resulting from analysis of brain homogenates, as applied to the physiology of the living brain, has always been the subject of much concern to workers in this field. Acetylcholine, the neurohumor most intensively studied, is a highly labile substance prone to rapid enzymatic and nonenzymatic destruction when in the free form. Furthermore, study of these substances in human beings usually precludes direct analysis of the brain. For these reasons, attention has been directed to CSF as a potential source of information in living animals. The fundamental assumption upon which such studies are based is that neurohumors liberated into the synaptic cleft may diffuse out into the surrounding area, and under certain circumstances, may be detected in CSF. It is apparent that it is a long road from synaptic cleft to CSF and many problems arise in interpreting such data. The most obvious of these is the lack of knowledge as to the specific site of origin of the neurohumor detected. Other questions to be raised pertain to the rates of appearance and disappearance of these substances in CSF. Monamine oxidase activity[59] and acetylcholinesterase are both present in CSF, presumably capable of acting on appropriate substrates.* Nonenzymatic destruction may also occur.

The site of withdrawal of CSF is also an important factor to consider in interpreting results. Many of the substances to be reviewed here have been found in ventricular and cisternal fluid but not in lumbar CSF. Histamine shows an opposite distribution as it is found in higher concentrations in lumbar fluid.‡ The reason for these variations is not entirely clear but is

* It appears that levels of acetylcholine which are usually found in CSF are too small to allow it to act as an effective substrate for acetylcholinesterase.[66] The same may be true of 5-hydroxytryptamine, since relatively large amounts of 5-HT are required to demonstrate monamine oxidase activity.[71] Thus the minute concentrations of neurohumors in CSF may protect them from enzymatic destruction.

‡ Actually most substances in CSF (glucose is an exception) are present in higher concentrations in a cranio-caudal direction.[81] For example, in central nervous system syphilis, ventricular fluid may show a negative Wasserman reaction when cisternal fluid will be weakly positive and lumbar fluid strongly positive.

probably dependent on the site of origin of the substance in question and the dynamics of CSF resorption.

In general, one might consider the study of CSF as complementary to analysis of brain tissue and as particularly suitable in situations where direct access to the brain is unobtainable.

"POSTERIOR PITUITARY-LIKE" SUBSTANCES (TABLE 1)

Prior to the extensive development of neurohumoral physiology, substances with oxytocic, pressor, antidiuretic and chromatophore-stimulating properties had been described in CSF. Many of the first workers thought these effects were due to posterior pituitary substance secreted into the CSF via the third ventricle. For this historical reason, the studies on 'posterior pituitarylike' substances have been considered first in this review.

Cushing and Goetsch[19] were the first modern workers to investigate the biological properties of CSF. They reported in 1910 that CSF obtained from human hydrocephalics caused a definite biphasic change in the blood pressure of a rat, initiated peristalis in the rat, and caused dilatation of the isolated frog's pupil. They speculated that these changes were due to pituitrin in the CSF occurring by virtue of the proximity of the third ventricle to the posterior lobe of the pituitary gland. This observation was not further pursued until the 1920's when other investigators began publishing reports along similar lines. Dixon[28] stated that the fluid obtained from the subcerebellar cistern of dogs "show every known chemical and physiological effect of pituitrin." Miura[46] and Trendelenburg[59] demonstrated oxytocic and melanophore-stimulating effects of CSF obtained from rabbits. Mestrezat and van Caulaert[48] stated that the oxytocic action was present in CSF obtained via cisternal puncture of dogs and humans with 'mental disease' but not when obtained by lumbar puncture. Hoff and Wermer[35] claimed that the oxytocic factor obtained from cisternal fluid of dogs, was much increased by exposing the dogs to cats prior to withdrawal of fluid. Karplus and Peczenik,[40, 41] in a series of carefully described experiments, reported that electrical stimulation of the hypothalamus or tuber cinereum of the cat caused an oxytocic substance to be released into CSF. Stimulation of the cervical sympathetic nerves or nervus ischiadicus did not elicit this effect. In addition to the oxytocic effect, a substance was also elicited that would darken the erythrophores of the minnow. They concluded that pituitrin and intermedin were liberated into the CSF under the influence of hypothalamic activity. This work may be considered as a forerunner of the present day intense interest in hypothalamic-pituitary relationships.

Van Dyke *et al.*[79] criticized the methodology of some of the above-mentioned papers and stated, on the basis of their own experiments, that the oxytocic effect of CSF was really due to its increased calcium concentration as compared to the usual perfusion fluids for guinea pig uterus. However, Cushing in his monograph *Pituitary Body and Hypothalamus*[18] reviewed the extant literature and decided that Van Dyke's conclusions were not valid. He states: "From the physiologic side . . . . the unmistakable presence in the CSF of a substance, which if not the active principle (of posterior pituitary) at least possesses similar properties, has since been clearly demonstrated by a number of workers."

Further investigations yielded conflicting results. Simon[62] and Friedman and Friedman,[30] using more refined techniques, were not able to confirm the presence of pressor or oxytocic activity in cisternal fluid of dogs or in concentrated lumbar fluid of humans. Volhard[80] studied 37 cisternal fluids from patients attending his hypertension clinic. Six of the 37 caused a contraction of guinea pig small intestine, but Volhard did not feel this was due to posterior pituitary substance. On the other hand, Barbour and Hambourger[5] said an oxytocic effect could be produced by submitting cats to a hot bath prior to cisternal taps while a cold bath would not accomplish the same effect! I. H. Page[56] demonstrated the presence of a highly active pressor substance in the fluid obtained post mortem from human cerebral ventricles, a substance much more potent than any present in lumbar fluid. Deleonardi[22] reported that the active substance obtained from cisternal fluid of rabbits or dogs, and from ventricular fluid of humans, showed oxytocic, antidiuretic, and pressor properties. Fluid obtained via lumbar puncture did not have these properties.

A particularly ingenious series of experiments was reported by Ungar in the years 1938-39.[72-75] He claimed that the oxytocic effect of cisternal fluid obtained from cats or dogs could be augmented or inhibited in several ways. Stimulation of the superior cervical ganglion and the peripheral end of the 2nd posterior spinal root caused a marked increase of the oxytocic effect, while stimulation of the cervical chain or the 3rd posterior spinal root produced no change.[73] Ungar related this to the pattern of autonomic innervation of the pituitary. Other devices for augmenting the oxytocic effect were shining a bright light into a cat's eye and direct stimulation of the optic nerve.[74]

Since 1940 there seems to have been little interest in further investigations of these phenomena. Van Dyke has never accepted the idea that posterior pituitary substances can be found in CSF.[77, 78] Recently, Scavo[68] reviewed the literature dealing with antidiuretic hormone in body fluids and

TABLE 1. "POSTERIOR PITUITARY-LIKE" SUBSTANCES IN CSF

Ref.	*Animal*	*Treatment or condition of animal*	*Assay method*	*Site of CSF withdrawal*	*Results*	*Comment*
Miura—49 and Trendelenburg—69	Rabbit		Guinea pig uterus Frog melanophores	Sub-occipital	Positive	Effects abolished by hypophysectomy
Barbour—5	Cat	Hot and cold baths	Guinea pig uterus	Sub-occipital ventricular	Cold bath—neg. Hot bath—pos.	
Ungar—72-75	Cats and Dogs	Various forms of stimulation	Guinea pig uterus	Sub-occipital	Positive	Level in cat higher than dog. Effects not abolished by atropine.
Dixon—23	Dogs		Guinea pig uterus	Sub-occipital	Positive	Substance water-soluble, insoluble in alcohol or ether, destroyed by trypsin but not pepsin.
Hoff—35	Dogs		Guinea pig uterus	Sub-occipital	Positive	Exposure to cats augmented effect
Karplus 40, 41	Dogs	Electrical stimulation of hypothalamus	Guinea pig uterus Erythrophores of minnow	Sub-occipital	Positive	
Simon—62	Dogs		Guinea pig uterus Guinea pig ileum	Sub-occipital	Negative	
Van Dyke—79	Dogs Humans		Guinea pig uterus	Sub-occipital ventricular	Negative	False-positive responses due to calcium content of CSF
Mestrezat—48	Dogs Humans	'Mental disease'	Guinea pig uterus	Sub-occipital lumbar	Positive Negative	
Friedman—30	Dogs Humans	Psychopaths	Rabbit uterine fistula (in vivo)	Sub-occipital lumbar (concentrated)	Negative	
Deleonardi—22	Rabbit Dogs Humans		Guinea pig uterus Blood pressure—cat Antidiuresis—rat	Ventricular sub-occipital lumbar	All assays positive All assays negative	Activity destroyed by warming 15 minutes with normal sodium hydroxide.
Cushing—19	Humans	Hydrocephalic	Blood pressure—rat	Ventricular	Positive	
Page—52	Humans	Post-mortem	Blood pressure—cat	Ventricular Lumbar	Positive Trace	
Volhard—80	Humans	Hypertensives	Guinea pig intestine	Sub-occipital	6—positive 31—negative	

concluded that the question of the presence of ADH in CSF is still unsettled.

In attempting to critically assess this array of contradictory reports that are usually not comparable, one is left with the feeling that pharmacologically active substances can be induced to appear in CSF by virtue of various stimulatory techniques. CSF obtained from ventricular or cisternal areas have higher concentrations of these substances than that obtained from lumbar sites. It seems impossible that all these effects could be due to artifacts in the experiments (e.g. high calcium content of perfusion fluid). However, it is entirely unclear as to whether these substances are related

TABLE 2. SOME PHARMACOLOGICAL EFFECTS OF NEUROHUMORS

	Oxytocic	*Pressor*	*Antidiuretic*
Acetylcholine	pos		pos
5-hydroxytryptamine	pos		pos
Histamine	pos		
Noradrenaline		pos	

in any way to the polypeptide hormones of the neurohypophysis. The activity of the neurohumors to be discussed subsequently may have substantially contributed to these findings as they were only beginning to be appreciated at the time most of this work was done. Acetylcholine antagonists, such as atropine, were usually not added to perfusion fluids although Unga briefly mentions that his effects were not abolished by atropine. He also states that his contractions were more persistent than those induced by either histamine or acetylcholine, but supplies no details upon which to evaluate his statements.[78]

Some pharmacological properties of the cerebral neurohumors are listed in Table 2 to illustrate how their presence could easily simulate posterior pituitary substances in CSF. Only the chromatophore-darkening effects cannot be explained in this way since neurohumors actually tend to oppose melanin stimulating hormone (lightening effect.)[44] However, adrenocorticotrophic hormone, which has also been reported in CSF (see below), has darkening activity similar to MSH. In general, one might suspect that many of the effects described above were due to neurohumoral substances in the CSF, but only further studies, utilizing our present knowledge of the properties of neurohumors, can satisfactorily resolve this problem.

ANTERIOR PITUITARY HORMONES

There are a few reports on the presence of anterior pituitary hormones in CSF. Van Bogaert and van Baarle[78] described a corticotrophic effect of CSF obtained from hypertensive dogs and humans. Lachnit and Weis[45] claimed that ACTH activity, as determined by ascorbic acid depletion in rats' adrenals, can be demonstrated in the CSF of patients with 'pituitary basophilism', while patients with primary adrenal disease did not show this phenomenon. They utilized this technique as an aid in differential diagnosis of these two conditions. Similarly, Soucek[68] reported that 3 out of 4 cases with Cushing's disease due to pituitary tumors had CSF that gave positive Aschheim-Zondek reactions. This was interpreted as indicating the presence of gonadotrophic hormone in CSF.

ACETYLCHOLINE (ACH) (TABLE 3)

Feldberg and Schriever[20] demonstrated in 1936, that the intravenous injection of eserine caused Ach to appear in the CSF obtained from the basal cisterns of dogs. Adrenaline and asphyxia increased the amount of Ach in CSF of these eserinized animals. These findings were subsequently confirmed by other investigators under various conditions. Chang *et al.*[18] stated that stimulation of the central end of the cut vagus nerve would cause increased Ach to appear in the CSF of eserinized animals. Adam *et al.*[1] described the same finding upon stimulation of the central end of several peripheral nerves. They also mention that while stimulation of the motor and cerebellar cortex is without effect, stimulation of the hypothalamus produced doubling of the Ach levels in 7 out of 14 animals studied. It is of some interest, in view of the reports summarized in the first section of this paper, that the authors state that perfusion of the ventricular system with pituitrin (10 units/cc) caused doubling of the Ach concentration, while perfusion with adrenaline had no effect.

Bornstein[10] in 1946 published a widely-quoted report on the presence and effect of Ach in CSF of cats subjected to experimental head trauma. He showed that Ach consistently appeared in CSF obtained by cisternal puncture of these animals in concentrations of 0.027-0.090 μgm/cc. The Ach could be demonstrated up to 48 hours after the trauma and then disappeared. The animals themselves initially showed tonic-clonic seizures, apnea, loss of placing reactions and finally stupor. Electroencephalograms revealed paroxysmal high-amplitude bursts of activity at first and then diminished activity with stupor. Bornstein stated that the EEG patterns and stuporous condition could be reversed by administration of atropine in doses of 0.5-1.0 mgm/kg although loss of placing reactions remained.

He also claimed that administration of intracisternal Ach duplicated both the behavioral and EEG picture of traumatized animals and could be suppressed with atropine. On the basis of this study, Ward[91] and later Jenkner[38, 39] gave anticholinergic drugs to patients with closed craniocerebral trauma and reported striking results in some instances, particularly with Jenkner's use of the centrally-acting phenothiazine 'diparcol.'

Frequent surveys have been conducted on patients with neurologic and psychiatric disorders for the purpose of searching for Ach in the CSF. Bioassay preparations used include the dorsal muscle of the leech,[34, 53, 55] the frog lung[9, 11 13] the frog abdominal rectus muscle,[24, 60] and most recently, the heart of the mollusc *Mercenaria (Venus) mercenaria.*[43, 57, 59, 60, 67, 70] These methods are all characterized by a considerable degree of sensitivity to Ach as concentrations reported are rarely higher than 0.1 μgm/cc. The frog lung is the most sensitive of these test organs, being able to detect Ach at a concentration of 10^{-10} gm/cc!

Earlier investigators found Ach in a wide variety of disease states—psychoses, general paresis, alcoholic polyneuritis and seizure states.[2, 11, 24, 50, 51] Brecht and Kummer[13] reported that Ach present in CSF of psychotic patients was not increased by electroshock therapy. Stüttgen and Jacob[84] studied CSF from 72 various sources and found an 'Ach-like' substance frequently. They state that a short period of heating at an acid pH increases the level of biologically active substances detectable in CSF. Tower,[66, 67] in one of the most extensive and carefully studied series, has stated that Ach is not present in normal CSF but is present in epilepsy up to 0.050 μgm/cc (average 0.010 μgm/cc) and following craniocerebral trauma up to 1.0 μgm/cc in severely involved patients. He found no association between the type of epilepsy and the amount of Ach present. Patients with positive CSF tended to have more extensive EEG changes and increased frequency of seizures. All specimens obtained during ictal and immediate postictal periods were positive for Ach. Many of the interictal specimens, however, were also positive. Anticonvulsant medication did not seem to affect the presence of Ach.

Poloni[65] in 1951 published a remarkable report regarding the absence of Ach in the CSF of schizophrenic patients. He studied the CSF of 110 patients with neuropsychiatric disorders and 10 normal individuals. He reported that the 10 normals and almost all of the patients except the schizophrenics, had Ach levels in their CSF ranging from 0.0001 to 0.1 μgm/cc. Forty-eight out of the 50 schizophrenics, however, had no detectable Ach in their spinal fluid. Other patients included "hysterics, epileptics, depressives, manics, senile dementia, alcoholics, and post-encephali-

TABLE 3. ACETYLCHOLINE IN CSF

Reference	*Animals*	*Treatment or condition of animal*	*Assay method*	*Site of CSF withdrawal*	*Results*	*Comment*
Feldberg—26	Dogs	Eserinized	Dorsal muscle of leech	Sub-occipital	Positive	
Chang—13	Dogs	Stimulation of central end of vagus	Dorsal muscle of leech	Sub-occipital	Positive	
Adam—1	Dogs and cats	Hypothalamic stimulation	?	Ventricular perfusate	Positive	Eserinized fluid used for perfusion
Bornstein—10	Cats	Head trauma	Frog rectus	Sub-occipital	.027-.090 μgm/cc	
Nochimkowski—51	Humans	Neuropsychiatric disease	Dorsal muscle of leech	Lumbar	32-cases—positive 34 cases—negative	9 cases of untreated general paresis—all positive
Brecht—11, 12	Humans	Meningitis, epilepsy, psychosis	Frog lung	?	10^{-10} to 10^{-9} μgm/cc (.001 μgm/cc)	Levels not increased by electroshock
Alcober—2	Humans	Neuropsychiatric disease	Frog lung	Lumbar	21 of 32 cases positive 10^{-10} gm/cc to 0.01 μgm/cc	
Tower—66, 67	Humans	Craniocerebral trauma Epilepsy	Clam heart	Lumbar	.002 to 1.0 μgm/cc .0002 to .05 μgm/cc	Ach not found in normal CSF
Stuttgen—64	Humans	Variable	Mouse small intestine(?)	?	'Ach-like' substance Average .080 μgm/cc	Pooled CFS studied
Poloni—55	Humans	Neuropsychiatric disease	Dorsal muscle of leech	Lumbar	.0001 to .1 μgm/cc	Positive in all but schizophrenics
Nielsen—50	Humans	Neuropsychiatric disease	Dorsal muscle of leech	Lumbar	21 of 27 cases positive	11 of 12 epileptics positive
Escalar—24	Humans	Neuropsychiatric disease	Frog rectus and leech muscle	Lumbar	10^{-12} to 10^{-11} gm/cc	Pre-treatment with trichlora cetic acid required for detection of Ach in CSF
Kunkle—42	Humans	Migraine and other neurologic disorders	Clam heart	Lumbar	5 of 9 migraine pts. positive; .004 to .06 μgm/cc	35 of 37 non-migraine negative
Sachs—57	Humans	Craniocerebral trauma	Clam heart	Lumbar	16 of 28 cases positive Up to .2 μgm/cc	
Turner—70	Humans	Neuropsychiatric disease	Clam heart	Lumbar and ventricular	See Table V	Ach stable in frozen CSF up to 2 years.

tics." At the same time, he describes a 'curare-like' substance present only in the CSF of schizophrenics and paretics, which had the effect of relaxing the leech muscle utilized for the assay. This substance antagonized Ach-induced contractions of the muscle but did not affect potassium-induced contractions. It was highly stable as opposed to Ach which required Tower's method of preservation in CSF (neostigmine plus acidification of CSF).[65] Manipulations that should have destroyed Ach did not affect this substance which Poloni measured as equivalent to 1.0-10.0 μgm/cc of d-tubocurarine. Recently, Turner and Mauss[66] published a report studying CSF from similar categories of patients which they interpret as not supporting Poloni's findings. They found ACh in amounts under 0.020 μgm/cc in roughly one-half of 59 fluids studied, but found no correlation between the presence of Ach and diagnostic groups. Ach was said to be found in schizophrenics in roughly the same frequency as other patients. This study cannot be interpreted as conclusively disproving Poloni's findings since only 11 lumbar fluids from schizophrenics were analyzed, yielding 4 samples positive for Ach. Poloni's report of curare-like activity in CSF was not investigated by these authors.

Kunkle[42] has reported Ach in CSF of 5 out of 9 patients with migraine headaches while no Ach was found in 35 of 37 patients without migraine. The two patients of this latter group who were free of migraine but did have Ach in their CSF were subject to grand mal seizures.

Bhattacharya, Feldberg, and Vogt[7] have further complicated matters by reporting a substance in human CSF that sensitizes frog rectus muscle to Ach which is demonstrated when the Ach is added subsequently. They feel that this agent may be a lipoprotein normally present in CSF. A similar phenomenon utilizing the clam heart as a test object has been noted.[50]

In reviewing these diverse reports of Ach in CSF, the same problems of comparability arise as with posterior pituitary-like substances. A few generalizations, however, can be formulated. Experimental electrical or chemical stimulation of the CNS in an appropriate manner, as reviewed above, will cause Ach to appear in the CSF of eserinized animals. Concentrations of Ach can be frequently found in CSF in many disease states, and may even be normally present in human CSF. The problem of deciding upon a normal range, and making valid clinical correlations with elevations above this, is almost impossible at this time. A rough approximation might indicate 0.001 μgm/cc as a dividing line under which investigators have frequently found Ach in CSF. Levels of 0.01 to 1.0 μgm/cc have usually been only found in certain neurological disorders, which at this time

include seizure disorders, craniocerebral trauma, and possibly migraine headaches.

Poloni's report of a curare-like substance associated with the absence of Ach in the CSF of 48 out of 50 schizophrenics seems particularly surprising in view of the current trend of thought that schizophrenia is a disease of multiple etiology. Although none of the other studies reviewed here indicated such an association, Poloni's claim has never been specifically disproved in the literature.

HISTAMINE

The first reported study of histamine in CSF was by Zadina and Petran[84] in 1938. They mention finding "very small amounts of histamine" in CSF of most patients studied and increased amounts in two cases of schizophrenia. Trabucchi[80] measured histamine in the CSF of 20 normal and schizophrenic patients. He stated that histamine was never present in concentrations over 0.01 μgm/cc in any of the cases and could not relate the height of the levels to the diagnosis.

In 1949, three different papers appeared almost simultaneously reporting data on determinations of histamine in CSF and reviewing the literature up to that time. Autio and Ermala,[3] using the guinea pig duodenum as their test object, found average levels of 0.043 μgm/cc in CSF of neuropsychiatric patients obtained by cisternal puncture, and 0.091 when obtained by lumbar puncture. This is an interesting finding in view of subsequent reports of unusually high histamine concentrations in dorsal root ganglia. They found that extraction of histamine with a conventional trichloracetic acid technique yielded higher values than when CSF was directly added to the test organ. Jackson and Rose,[37] using a similar method, report much lower levels in CSF, averaging 0.01 μgm/cc with a range of 0.002 to 0.03 μgm/cc. The highest level was found in a case of trigeminal neuralgia. No associations were made, other than that patients with high CSF cell counts tended to have higher histamine levels. Corrado[16] as well as Stüttgen and Jacob,[64] utilizing raw CSF on guinea pig ileum, reported average levels of 0.025 μgm/cc and 0.045 μgm/cc, respectively. None of these authors could significantly relate the histamine levels to the presence of psychiatric or neurological disorders.

Baccarini and Volpicelli[4] have further indicated the association of CSF histamine with the cellular elements in CSF by reporting, in a small group of cases (seven), that the sediment of centrifuged CSF generally contained most of the measurable histamine.

Many of the recent studies of histamine in CSF have the virtue of utilizing methods which involve specific extraction of histamine prior to bioassay,[3, 4, 87] rather than the use of raw CSF as has often been used in studies of other pharmacologically active substances. Even so, the average levels reported vary considerably among different investigators (0.01 to 0.09 μgm/cc). Histamine seems to be constantly present in CSF, but all workers since Zadina and Petran agree that the levels are not related to the presence of neuropsychiatric disease.

CATECHOLAMINES (ADRENALINE AND NORADRENALINE)

Only two reports have been found describing catecholamines in CSF. Although these utilized chemical rather than biological methods, they have

TABLE 4. CSF LEVELS OF CATECHOLAMINES (WEIL-MALHERBE)

	Organic brain lesions	*Chronic schizophrenia*
Adrenaline	0.9 μgm/liter	0.93 μgm/liter
Noradrenaline	2.46 "	3.13 "

been included here because catecholamines are usually listed in classifications of neurohumoral agents and have a high degree of pharmacological activity. Weil-Malherbe and Liddell,[88] using the ethylene diamine condensation method, reported adrenaline and noradrenaline levels in the CSF of patients with organic brain lesions and with schizophrenia (Table 4). The levels generally are proportional to, although less than, those found in plasma using the same method. Manger *et al.*[47] using a modification of the Weil-Malherbe method, subsequently reported that total epinephrine-like substances in CSF average 0.8-1.2 μgm/liter.

The methodology of catecholamine determinations, utilizing colorimetric or fluorimetric techniques, is at present quite controversial; hence, measurements of the small concentrations found in CSF must be regarded with considerable caution. However, in view of the sensitivity of the 'reticular activating system' to adrenaline,[9, 21, 86] this area has much promise if appropriate assay techniques can be developed.

5-HYDROXYTRYPTAMINE (5-HT, SEROTONIN)

Reports of 5-HT in CSF are of quite recent origin. In 1957 Sachs[81] first reported this substance to be present in CSF of patients following craniocerebral trauma. He utilized as his test object, the clam heart *(Venus*

mercenaria), a preparation that is particularly useful in surveys of CSF because it is highly sensitive to both 5-HT and Ach. 5-HT produces increased contractility of the heart while Ach produces the opposite effect. Positive identification of these substances is usually felt to be reasonably acceptable with the use of specific antagonists such as lysergic acid diethylamide (LSD) for 5-HT and benzoquinonium ('Mytolon') for Ach. Sachs found 5-HT as well as Ach in a number of the patients he investigated, thus enlarging upon Tower's earlier reports that Ach appears in the CSF of patients undergoing head trauma.

Bogdanski, Weissbach, and Udenfriend,[8] as part of their pharmacological analysis of 5-hydroxytryptophan, reported that 5-HT appears in the CSF of cats and dogs up to levels of 0.14 μgm/cc following administration of

TABLE 5—DATA FROM TURNER AND MAUSS

		Ach		*5-HT*	
Origin of CSF	*Total no. of fluids*	*No. of positives*	*Av. level μgm/cc*	*No. of positives*	*Av. level μgm/cc*
Ventricular	15	11	0.006	8	0.012
Lumbar	44	20	0.0056	9	0.006

5-HTP (a precursor of 5-HT). The authors have shown that brain 5-HT increases up to tenfold, and is associated with marked behavioral and autonomic changes which they interpret as resembling an LSD effect. The significance of these findings awaits an understanding of the role played by 5-HT in the brain.

Feldtman, Izak, and Nelken[20] could not find significant amounts of 5-HT in CSF of patients with multiple sclerosis and other neurological disorders. Turner and Mauss[70] have reported an extensive series of cases, as mentioned in the Ach section, utilizing the clam heart in the same way as Sachs did. They studied raw spinal and ventricular fluids from subjects with schizophrenia and a variety of neurological conditions (see Table 5). 5-HT was found in about one-third of all fluids studied. No significant correlations could be made between the levels found and the diagnostic groups studied. It appears that there is a tendency for ventricular fluids to have higher 5-HT levels, although the authors did not feel that this was a significant difference.

Freedman, Giarman, and Picard-Ami,[29] testing a 95% acetone extract of CSF on the clam heart, have been able to detect on only rare occasions

minute amounts of 5-HT (under 0.01 μgm/cc) in the CSF of schizophrenic patients. Schain[59] studied a group of 30 specimens of raw lumbar CSF from subjects with various neuropsychiatric disorders, using the clam heart and the rat uterus in estrus. 5-HT could be definitely identified on only one occasion (infantile autism). It has been noted that most CSF stabilizes and increases the height of the base-line contraction of the heart. Subsequent to this effect, many hearts become more sensitive to additions of the standard solution of 5-HT. These phenomena may often hinder the assayist from accurately assessing the presence and level of 5-HT in CSF.

The levels of 5-HT in CSF reported by Turner and Mauss tend to approach the lower limits of sensitivity of the clam heart to 5-HT by the usual assay methods. Some doubt must be expressed as to whether levels under .005-.01 μgm/cc can be accurately identified as 5-HT. Furthermore, data obtained by techniques utilizing relatively purified extracts of 5-HT, may be looked upon with considerably more confidence than those derived from raw body fluids. This problem of the use of raw fluids in bioassay procedures will be discussed again below.

POLYPEPTIDES

Chapman and Wolff[14, 15] have reported the presence of a proteolytic enzyme in the CSF of patients with active inflammatory or degenerative central nervous system disease, migraine headaches, prolonged pain, and chronic schizophrenia. This protease is identified by virtue of its capacity to form what appears to be a biologically-active polypeptide (bradykinin) when CSF is incubated with a globulin-containing solution. The polypeptide is assayed on rat uterus or guinea pig ileum. Sometimes the CSF seems to contain the polypeptide even without incubation with globulin which the authors attribute to the action of the protease on CSF globulins *in vivo*.

Many biologically active polypeptides have been detected in body fluids and tissues, and have been the subject of much investigation.[61] One of them, Substance P, is found in appreciable amounts in the brain and has been considered as a possible neurohumoral transmitter. Posterior pituitary substance mentioned previously as frequently reported in CSF, is now known to be a mixture of several polypeptides with different pharmacological effects that are found in the neurosecretory elements of the hypothalamus and the hypothalamic-hypophysed tract. The possible relationship of local neurosecretion to brain function is of much interest but beyond the scope of this review.

DISCUSSION

The chaotic mass of data reviewed above does not lend itself readily to critical analysis. Furthermore, the fact that there is no real understanding of the normal role played by neurohumors in the CNS complicates the problem still more. Nevertheless, the author feels that investigating the patterns of neurohumors in CSF offers a parameter for study that may directly reflect the dynamics of brain function, and should be pursued in spite of the lack of clarity of the field.

The data reviewed on 'posterior pituitary-like' substances in CSF clearly indicate that an agent (or agents) with diverse pharmacological effects appears in increasing concentration in CSF when certain CNS stimuli are applied. Reinvestigation of this problem should be attempted in light of our extensive knowledge of the properties of neurohumors as well as polypeptide hormones.

The presence of measurable Ach in CSF has been clearly established in many experimental and clinical situations, although the levels found vary greatly. The association of increased Ach levels with craniocerebral trauma and epilepsy seems definite. It has been felt that this represents a massive release of Ach from neurons secondary to trauma or seizure activity. This is certainly true of trauma, but one might speculate whether epileptics are frequently releasing excessive amounts of Ach that is a sign of their predisposition to seizures. The fact that elevated Ach levels have been found in the CSF of epileptics during interictal periods[66] would support such a hypothesis. There is some evidence that convulsions produced by electroshock therapy do not produce Ach elevations as readily as naturally occurring seizures do.[13, 97] The data on choline acetylase activity in epileptogenic cortex has been contradictory,[54] but the increasing emphasis at present on techniques of studying particulate (bound) versus free Ach in the brain[84] might help resolve this problem. Perhaps Ach in CSF reflects convulsive threshold as well as convulsive activity.

Bornstein[10] and Feldberg and Sherwood[27] have shown that cisternal or intraventricular administration of Ach to cats produces, among other effects, a dazed or stuporous reaction. One wonders whether some of the symptomatology of the postictal state is due to Ach in CSF.

The biogenic amines have been intensively investigated for the past decade, particularly with respect to their possible relationship to mental illness. There has been no evidence presented to indicate that abnormal CSF (or brain) levels are present in mental or nervous disease. It may be that these substances are not normally released into the synaptic cleft, as Ach probably is, and are therefore less likely to diffuse out into CSF.

Catecholamines in CSF undoubtedly reflect plasma levels of these substances. 5-HT in blood is almost entirely bound to platelets, and it is not surprising that 5-HT is not as readily detectable in CSF as are catecholamines. Where 5-HT is found, it would be worthwhile to study the sediment of centrifuged CSF to see if the cellular elements are the source of 5-HT, as seems to be often true of histamine.[4]

A few other suggestions regarding further investigations of this type may be made. Although the surveys of heterogeneous patient groups described above are initially necessary, continued advances will now probably come as the result of sustained studies of specific pathological states where promising leads have been found. One such area is the association of Ach in CSF with convulsive disorders. The study of psychomotor seizure states might be a particularly fruitful type of fit to study in view of the high levels of neurohumors found in certain regions of the 'limbic system.'[52] If 5-HT from neural sources were ever to make its appearance in CSF, one might think this should occur during a psychomotor seizure originating from an area rich in 5-HT. It would be of interest to correlate CSF findings with EEG studies in the so-called 'epileptic equivalent' states, e. g. abdominal pain, headaches, nightmares, etc., that are thought to be at times the manifestations of seizure disorders. Identification of these symptoms as epileptic equivalents is often quite difficult, and evidence of abnormal Ach or other neurohumoral levels in CSF would be of much interest.

Poloni's provocative report has not been mentioned again in the literature, except by Turner and Mauss, as far as the writer has been able to determine. His findings should be either confirmed or disproved, for if true, they would have major implications regarding a neurohumoral abnormality in schizophrenia.*

The study of neurohumors in CSF in association with the administration of drugs is a second approach that might provide pertinent information regarding the mechanism of action of neurotropic drugs. Evidence is accumulating that these preparations exert their effect by acting on neurohumoral mechanisms. It would seem reasonable that administration of such drugs might be associated with the appearance of neurohumors in the CSF or with changes of levels already present. The observation of Bogdanski *et al.*,[8] as mentioned above, is an example of evidence that administration of a precursor of 5-HT causes the latter to appear in CSF with the simul-

* Poloni has described other pharmacologically active substances in CSF. He characterizes CSF from epileptics as having 'antidiuretic, adrenocorticotrophic and epileptogenic' properties. CSF from schizophrenics has 'anti-epileptogenic, diuretic and curare-like' properties. This work is summarized in Poloni, A., *Riv. neurol.*, 1954, *24*, 863.

taneous development of marked behavioral changes. A well-known older observation is the fact that administration of an anticholinesterase such as eserine, causes Ach to appear in the CSF.[13, 26] Possibly other enzymatic inhibitors such as monamine oxidase inhibitors, would cause other neurohumors to appear in CSF in an analogous fashion. Drugs such as reserpine that are thought to decrease the binding capacity of subcellular particles for amines, might produce similar effects. Speculations of this sort could be easily tested by the analysis of CSF in drug-treated animals.*

Cisternal CSF should be obtained more often, rather than relying exclusively on lumbar CSF, since the former may yield positive data where lumbar fluids are negative. Unfortunately, cisternal puncture is rarely attempted in this country or is generally regarded as a dangerous procedure. Actually, this technique is widely used in continental Europe and Schonenberg states that suboccipital (cisternal) puncture is the procedure of choice for obtaining CSF in all but young children or infants.[61] He reviews the literature and concludes that, while unfortunate sequelae have been reported, they are extremely rare.

A final point that should be emphasized is the necessity for rigorous technique in the use of bioassay procedures. Much of the difficulty with these techniques is due to inadequate separation (or no separation at all) of contaminating tissue factors from the substance to be measured. It is well-known that tissue extracts commonly exert sensitizing effects on biological preparations,[25, 48] and it has been observed above that an unknown substance in CSF will sensitize the response of test organs to Ach and 5-HT. The use of specific pharmacological antagonists for identification is only a partial answer as the antagonists themselves may exert prolonged effects on the test object and will slow down an already time-consuming procedure. Increased emphasis on extraction methods would do much to improve the reproducibility of bioassay results.

Regarding the test object itself, the same biological preparation will vary considerably over a period of time in its sensitivity to active substances. The usual bioassay method requires the bracketing of the unknown between two standards that demonstrate clearcut dose differentiation.* Preliminary 'testing of the sensitivity' without continual use of the standard solution is

* Bhattacharya and Feldberg's technique of slowly perfusing the ventricular system of cats and collecting the effluent from the cisterna magna or aquaduct,[9] might provide a continual source of CSF useful for investigating the influence of neurotropic drugs in releasing pharmacologically active substances into CSF.

* Other, more refined, techniques are also available if greater degrees of accuracy are required, such as in determining drug potency (Burn, J. H., Finney, D. J., and Goodwin, L. G.: *Biological standardization*, 2d ed. London, Oxford University Press, 1950).

a quite unreliable procedure, particularly when using a raw body fluid such as CSF. The significance of the data obtained from bioassay techniques, as in any other laboratory procedures, is directly related to the care taken in attempting to obtain quantitative analysis.

SUMMARY

1. The literature dealing with pharmacologically active substances in CSF has been reviewed. Many earlier reports described oxytocic, pressor, antidiuretic and chromatophore-stimulating agents in CSF. These cannot be considered as all artifacts, but the identity of the active principles has not been determined.

2. Acetylcholine is the only pharmacologically active substance of clearly neural origin, that has been specifically identified in CSF. Craniocerebral trauma and seizure states are associated with higher levels of acetylcholine in CSF.

3. Histamine, catecholamines and 5-hydroxytryptamine have all been reported in CSF, but it cannot be assumed that these substances in CSF originate from neural tissue.

4. Certain suggestions for further research have been discussed. These primarily deal with the study of CSF neurohumors in seizure disorders, and following the administration of neurotropic drugs.

5. Bioassay methodology has been discussed. Much stress has been placed on the importance of using extraction methods prior to assay. The principles and some pitfalls of bioassay technique have been briefly mentioned.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. Daniel X. Freedman and to Dr. Nicholas Giarman for generously making their laboratory facilities available to me and offering much advice and encouragement.

The author is indebted to Dr. Giancarlo Pepeu who provided translations of the papers from the Italian literature as well as informed opinions of the data contained therein.

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ROBERT W. CHAMBERS†

Departments of Pathology, Emory University School of Medicine, and of Internal Medicine, Yale University School of Medicine

ADENOHYPOPHYSEAL CHANGES IN PATIENTS DYING OF ACUTE RENAL TUBULAR NECROSIS‡

A method of studying the topographical distribution and cytological characteristics of the glandular cells of the human adenohypophysis was described in a previous report.[13] A cell classification based on probable functional significance was introduced in an attempt to achieve a greater degree of correlation between morphological and clinical observations. Application of this method to a group of normal adult and male adolescent glands revealed a predominance of storage cells in the adult, while adolescent glands showed evidence of increased secretory activity in both basophilic and acidophilic cells. These alterations, it was felt, reflected increased pubertal gonadotropic and growth hormone secretion.

The present study is an evaluation of adenohypophyseal cytology in patients dying in uremia of acute tubular necrosis. The striking alterations observed in both acidophilic and basophilic cells are interpreted as the morphologic expression of altered pituitary function in response to acute malnutrition. Patients with acute renal disease warrant special consideration because all were in good health until the onset of renal failure and their clinical course was short and could be studied in detail. They represent, furthermore, the special problem of possible effects on the hypophysis of renal disease per se.

METHODS

The method of study of the adenohypophysis has been described in detail elsewhere.[13] Following fixation in buffered 10 per cent USP formalin, the glands were cut so as to remove first a 1 mm. mid-sagittal section including the pituitary stalk. The remain-

* Professor of Pathology, Emory University School of Medicine.
** Associate Professor of Medicine, Yale University School of Medicine.
† Fourth-year medical student, Emory University School of Medicine.
‡ This work was aided by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service, (A-1719). The technical assistance of Mrs. Elizabeth Fortson is gratefully acknowledged.

Received for publication 23 February 1960.

ing portions were then bisected, one in the equatorial, the other in the coronal plane. Paraffin sections from all glands were stained with Pearse's trichrome PAS,[29] Mallory's orange G-aniline blue,[24] Gomori's aldehyde fuchsin,[16] Halmi's aldehyde fuchsin counterstained with orange G-fast green,[15] and Mallory's phosphotungstic acid hematoxylin.[24]

The trichrome PAS sections were employed for a quantitative study of the topographic distribution of cell types. Contiguous "high-dry" microscopic fields were examined so as to cover an entire histologic section. A differential count, continuous from field to field, was performed, recording one score for approximately 25 cells of each type. The data were then transferred to squared graph paper, with each square representing one microscopic field, one dot on the graph representing approximately 25 cells.

The following cell classification was employed:

Storage basophils. Cells with small nuclei whose cytoplasm was densely granulated with PAS positive material.

Storage acidophils. Cells with small nuclei whose cytoplasm was densely granulated with orangophilic material.

Secretory basophils. Large cells with generally prominent nuclei and delicate cytoplasmic stippling by fine, predominantly PAS positive granules.

Secretory acidophils. Large cells with cytoplasmic stippling by predominantly orangophilic granules.

Hypersecretory cells. Cells with very large, hyperchromatic or vesicular nuclei and variable cytoplasmic mass containing only occasional PAS positive or orangophilic granules.

Chromophobes. Cells with small nuclei and scant agranular cytoplasm.

Many of the secretory cells were truly amphophilic, containing both PAS positive and orangophilic cytoplasmic granules. They were classified according to their predominant granulation.

Sections of the other organs, including the endocrine glands, were fixed in Zenker's fluid with 5 per cent glacial acetic acid, embedded in paraffin and stained with phloxine and hematoxylin. Adrenal sections were also stained by Wilder's reticulum method.[48]

RESULTS

Clinical observations. Six patients dying in uremia of acute renal tubular necrosis were studied. There were five females and one male with ages ranging from 29 to 75 years. The periods of illness varied from 7 to 21 days and the patients were observed at Emory University Hospital for from 3 to 15 days. Tubular necrosis occurred as a complication of surgical procedures in three patients and in the other cases was related to (i) placenta accreta with ruptured uterus, (ii) acute cholecystitis, and (iii) undiagnosed acute febrile disease.

The significant clinical observations are described in the Appendix, and are summarized in Table 1. All six patients were uremic during the period of observation. Death appeared to be due directly to uremia and associated

TABLE 1. CLINICAL OBSERVATIONS IN PATIENTS DYING IN UREMIA OF ACUTE TUBULAR NECROSIS

Case	*Age*	*Sex*	*Precipitating factors*	*Total illness (days)*	*Days at Emory Hospital*	*Est. daily caloric intake* during observation*	*Average urine output ml/day*	*Hormonal therapy*
1	38	F	Hysterectomy, oophorectomy, bilateral. Transfusion reaction	14	11	250	50 (35-125)	300 mgm. Cortisone 14 days before death
2	75	F	Acute cholecystitis	9	5	700	0	Insulin (with glucose)
3	37	F	Ruptured uterus; placenta accreta	15	6	700	25 (0-60)	Oral thyroid for 15 months (? dose)
4	29	F	(Obscure) Chills & fever	21	15	975	870 (50-3000)	ACTH Insulin (with glucose)
5	68	M	Transurethral prostatectomy Shock	13	5	220	170 (90-500)	Insulin (with glucose)
6	48	F	Subtotal gastrectomy	7	3	500	0	None

* The only source of calories was glucose.

electrolyte disturbances in four patients, to congestive heart failure in one (Case 5), and to bronchopneumonia in another (Case 6). Five patients remained severely oliguric or anuric. One patient (Case 4) had a copious diuresis, but continued to have an elevated non-protein nitrogen and developed hypokalemia.

All patients were maintained on either intravenous glucose alone, or combined intravenous and oral carbohydrate solutions. The estimated average daily caloric intake ranged between 220 and 975. These estimates represent total intake and do not take into account losses caused by vomiting or gastric suction.

Three patients received injections of insulin. One patient (Case 4) was given adrenocorticotropin throughout her hospital course, and another (Case 1) received 300 mg. cortisone the day of onset of illness. Patient 3 had been taking an unknown thyroid preparation for 15 months.

Morphological observations. The adenohypophyses of all of these patients showed striking changes. These consisted of a marked increase in the proportion of secretory acidophils and hypersecretory cells, and, with one exception, a decrease in secretory basophils.

Table 2 shows the results of the differential counts performed on the equatorial sections of the glands. Secretory acidophils, which normally comprise 1 to 3 per cent of all cells, here accounted for 17.7 to 37.0 per cent. Hypersecretory cells, virtually absent from normal glands, represented 7.2 to 16.1 per cent of all cells. The marked shift to secretory acidophils and hypersecretory cells appeared to be at the expense of both storage acidophils and chromophobes. The proportion of secretory basophils was markedly decreased (1.7 to 2.8 per cent) in five patients. They were increased above the normal range in one patient (Case 1), a 38-year-old female who had undergone bilateral oophorectomy 14 days before death. Differential counts of the mid-sagittal and mid-coronal planes showed no significant variation from those of the equatorial sections.

The topographical distribution of cell types in Patients 2 and 3 are shown in Figures 1 and 2. A typical cell distribution pattern from a normal adult adenohypophysis is included for comparison (Fig. 3). The illustrations emphasize the striking increase in the proportion of secretory acidophils. These tend to be most concentrated in the peripheral portions of the sections. Hypersecretory cells are also prominent in these glands and show a distribution similar to that of the secretory acidophils.

The distribution of cells in the other hypophyses did not vary significantly from those illustrated. The distribution of the increased numbers of secretory basophils in Patient 1 paralleled quite closely the distribution of

TABLE 2. PERCENTAGE DISTRIBUTION OF ADENOHYPOPHYSEAL CELLS IN ACUTE TUBULAR NECROSIS
(Equatorial Plane—Trichrome—PAS stain)

Case	*Age*	*Sex*	*Storage basophils*	*Secretory basophils*	*Storage acidophils*	*Secretory acidophils*	*Hypersecretory cells*	*Chromophobes*
1	38	F	16.0	13.5	31.6	17.7	13.1	8.1
2	75	F	12.6	2.8	20.8	37.0	16.1	10.7
3	37	F	16.0	1.7	36.1	29.8	7.2	9.2
4	29	F	12.5*	1.7	38.3	29.8	10.0	7.7
5	68	M	4.2	2.0	46.9	31.4	10.1	5.4
6	48	F	13.9	2.9	28.9	33.9	13.2	7.2
	Normal Adult Range (13)		11-17	6-10	44-52	1-3	±0	22-35

* Crooke's hyaline change noted in many of these cells.

storage basophils. Many of the storage basophils in Patient 4, who had received adrenocorticotropin therapy, displayed Crooke's hyaline change.[15, 23]

Preparations stained by the method of Halmi showed some variation from gland to gland in the relative numbers of "beta" and "delta" cells but there was no significant variation in different areas of any one gland.

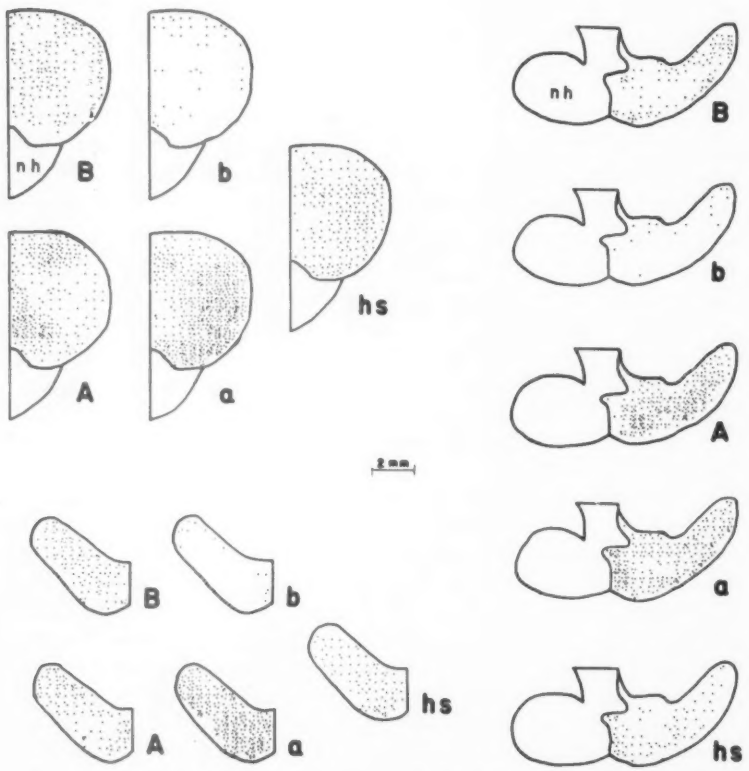

FIG. 1. Topography of adenohypophyseal cell distribution in Case 2, sectioned in mid-sagittal, equatorial, and coronal planes. The upper left group shows the equatorial plane. The lower left group represents the coronal plane with the upper margin of the section corresponding to the upper extreme of the graphs. The group at the right shows the mid-sagittal plane. *B*. Storage basophils. *b*. Secretory basophils. *A*. Storage acidophils. *a*. Secretory acidophils. *hs*. Hypersecretory cells. *nh*. Neurohypophysis. Each dot represents approximately 25 cells. Note the large numbers of secretory acidophils and hypersecretory cells which tend to be most concentrated at the peripheral areas of the sections. Note also the paucity of secretory basophils. Compare with Figure 3.

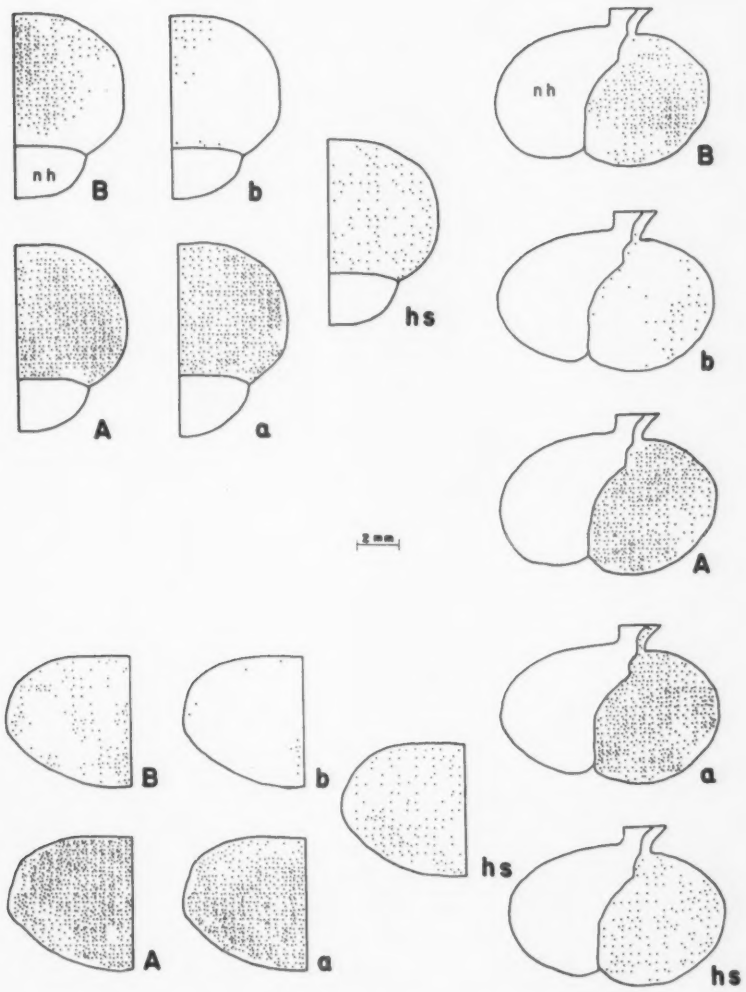

FIG. 2. Topography of cell distribution in Case 3. Symbols and orientation as in Figure 1. The distribution of cells is very comparable to that in Figure 1.

The adrenal glands of these patients were within normal weight range except for Patient 4 who had received adrenocorticotropin therapy. There was marked lipoid depletion of the cortices in five of the six patients. The thyroid glands showed cuboidal to low cuboidal follicular epithelium and normal appearing colloid. Several glands showed mild interstitial fibrosis and in one there was slight interstitial chronic inflammatory cell infiltration. The gonads were absent in one patient, normally active in two, and atrophic in three. Parathyroid glands were examined in two patients. They appeared hyperplastic in one, and normal in the other. The pancreatic insular tissue was normal in all patients.

DISCUSSION

The adenohypophyses of six patients dying in uremia of acute renal tubular necrosis showed striking morphological alterations compared with the normal gland (Table 2). All of them showed a markedly increased proportion of cells characterized cytologically by large active nuclei and cytoplasm containing delicate acidophilic granules *(secretory acidophils)*. Still more active cells with hyperchromatic nuclei and very little visible cytoplasmic granulation *(hypersecretory cells)* comprised a significant percentage of all cells. The augmentation of these two cell types was at the expense of densely granulated *(storage)* acidophils and small inactive chromophobes. Delicately granulated cytologically active basophilic cells *(secretory basophils)* were reduced in proportion in five of the six glands.

The interpretation of morphological changes in terms of altered physiological activity is difficult and may be misleading. The classification of cell types used in the present study is based on alterations which have been described in secreting cells of other glands, and on the assumption that such changes have a comparable significance when observed in the pituitary gland. In addition, the designation of small, densely granulated cells as "storage" cells and the larger, cytologically more active and sparsely granulated cells as "secretory" elements is supported by many observations in patients with clinical evidence of altered hypophyseal function, particularly those of Severinghaus,[40] Pearse,[80] Russell,[68] and Russfield.[67] Our findings in the present group of hypophyses are therefore interpreted as showing a marked increase in the secretory activity of the acidophil cells and decreased secretory activity of the basophils. Since the weight of evidence indicates that acidophils secrete growth hormone[6, 12] and that basophils secrete adrenocorticotropin,[25] thyrotropin, and gonadotropins,[29, 30] we would infer on the basis of morphological observations that these patients were secreting in-

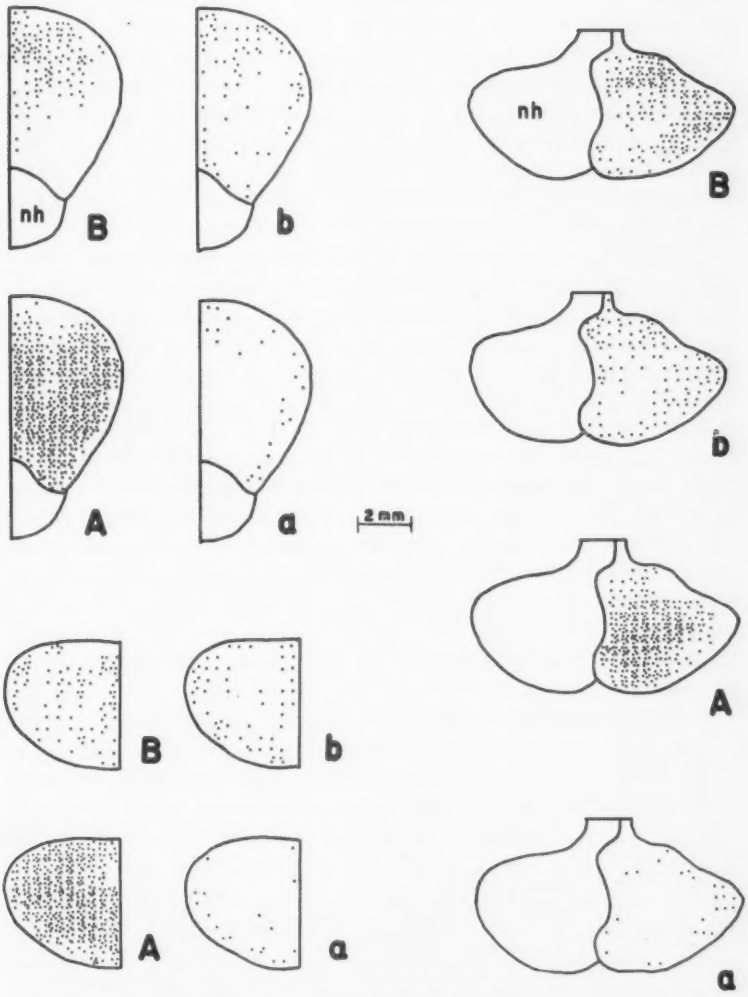

FIG. 3. Cell distribution in a normal adult male who died of acute carbon monoxide poisoning. Symbols and orientation as in Figure 1. Note predominance of storage acidophils, the small numbers of secretory acidophils, and the greater number of secretory basophils when compared with Figures 1 and 2. Hypersecretory cells were not present.

creased amounts of growth hormone and decreased amounts of the hormones of basophil origin.

Support for these conclusions must be sought in the physiological status of our patients. Although they differed in many respects, certain important disturbances were common to all. After the onset of renal failure they were severely undernourished in terms of calories, and absolutely starved of proteins and fats. All showed progressive renal failure with extensive tubular destruction, uremia and electrolyte disturbances. Finally, all had been acutely ill at the onset of their disease.

The effects of starvation may be considered first. The metabolic pattern during caloric deprivation shifts to one in which fat becomes almost the only substrate, while protein and carbohydrate are spared. This adjustment may depend to some extent on the secretion of growth hormone. Growth hormone (along with corticotropin and thyrotropin) is known to promote ketogenesis[1, 10] and the mobilization of fat from the depots both as free fatty acids[11, 22] and as triglycerides which move to the liver.[9, 17, 20] At the same time, growth hormone reduces the breakdown of protein[5, 42] and limits the utilization of carbohydrate both directly and by promoting its storage as muscle and liver glycogen.[19, 35, 36] Since the metabolic adjustments to starvation are parallel to those produced by growth hormone, it has been suggested that the secretion of this hormone may be an important factor in the adjustments to caloric restriction.[24] The administration of carbohydrate supplements (as in our patients) would not be likely to alter this response qualitatively, although it would enhance the sparing of protein and reduce the severity of ketosis.

A further stimulus to growth hormone secretion could have been the reduced mass of functioning renal tissue in these patients. It is well established that compensatory renal hypertrophy is greatly reduced in the absence of the pituitary,[3] and that pituitary growth hormone is a potent renotropic substance.[41] The stimulus to renal hypertrophy appears to be the reduction of renal functioning mass rather than uremia itself.[7]

These physiological considerations lead to the conclusion that the increased numbers of secretory acidophils and hypersecretory cells observed in the adenohypophyses of these patients might well represent the morphological expression of enhanced secretion of growth hormone.

An interpretation of the decrease in secretory basophils requires consideration of factors which may have modified adrenal cortical, thyroid, and gonadal function in these patients. These include the effects of starvation, acute stress, and the loss of excretory function.

Undernutrition, whether in the form of anorexia nervosa[6] or in the enforced starvation of prisoners of war,[20, 29] leads to depression of gonadotropic hormone secretion. Starvation is also associated with reductions in basal metabolic rate and thyroidal iodine uptake[5, 23] and histological changes in the thyroid consistent with reduced function.[20] The secretion of thyroid-stimulating hormone in response to goitrogens is reduced in starving animals.[26]

Adrenal function is probably reduced in starving humans, since the excretion of total 17-ketosteroids[23] and of 11-oxygenated 17-ketosteroids[25] diminishes. The plasma 17-hydroxycorticosteroids remain normal or even elevated,[20, 25] probably because the rate of catabolism of the steroids is reduced. Furthermore, the secretion of ACTH may be limited by the absence of an adequate protein intake.[27]

One might expect the acute onset of illness to have been associated with an increased secretion of ACTH. By the time of death, however, renal failure and impaired hepatic function (which often accompanies the shock leading to tubular necrosis) would probably have reduced the rate of destruction and excretion of adrenal hormones so that a high plasma steroid concentration would be maintained even though adrenal activity returned to a normal level.[28] The loss of excretory function might similarly contribute to a reduction of gonadotropin and thyrotropin secretion.

The reduced percentages of secretory basophils in the adenohypophyses of our patients, therefore, are consistent with reduced secretion of gonadotropins, thyroid-stimulating hormone, and ACTH during the terminal phases of illness. The morphological observations of the target organs in these patients are compatible with this conclusion. Case 1, however, is an interesting exception, since secretory basophils were increased above the normal range. This patient had had a bilateral oophorectomy just before the onset of renal failure, and the increased percentage of secretory basophils probably represented the effects of castration.[4]

Our interpretation of the morphological changes in the adenohypophyses of these patients appears to be supported by a consideration of their nutritional status and its expected effects on pituitary function. The patients, however, were also suffering from renal failure and some had additional disturbances such as congestive heart failure and infection, disturbances which could have contributed to altered pituitary function. The only constant finding, aside from undernutrition, was an elevated blood urea nitrogen. Observations of the pituitary morphology of undernourished patients without uremia would separate the role of these two factors in causing the acidophilic changes described in this report. Preliminary observations of

autopsy material from severely undernourished patients dying of cancer show changes comparable to those described in the uremic patients, whereas the cytological pattern of patients who had maintained their nutritional status deviates only slightly from normal.[14]

We conclude that the morphological changes observed in the adenohypophyses of our group of patients with renal failure reflect altered hypophyseal function, possibly due to malnutrition. It is suggested further that nutritional status may be a significant factor that should be taken into account in the evaluation of all human pituitary glands.

SUMMARY

The adenohypophyses of six patients dying in uremia of acute renal tubular necrosis were found to contain an increased proportion of secretory acidophils and hypersecretory cells and decreased percentages of secretory basophils. The increased secretory activity of the acidophil cells is consistent with the concept of increased growth hormone secretion and may reflect a response to starvation. The decrease in secretory basophils probably indicates a diminished secretion of gonadotropins, thyroid-stimulating hormone, and adrenocorticotropin. These observations indicate that the nutritional status of the patient should be taken into account in the evaluation of human pituitary cytology.

APPENDIX

Case 1. This 38-year-old female underwent hysterectomy and bilateral salpingo-oophorectomy three days before transfer to Emory University Hospital. She received two units of incompatible blood during the operation and was in shock for 28 hours. Hemoglobinuria was followed by severe and persistent oliguria. She was given several further blood transfusions, pressor agents, and 310 mg. of cortisone to combat shock. Paralytic ileus required Wangensteen suction.

The daily urinary output varied between 35 and 125 ml. during the 11 days of observation at Emory University Hospital. Hemodialysis was performed on the 4th and 11th hospital days. The temperature varied between 98.0° and 101.2° F. The terminal plasma non-protein nitrogen was 255 mg./100 ml., the serum sodium 125 m.Eq./L., the potassium 6.7 m.Eq./L., and the blood hemoglobin 10.8 gm./100 ml.

The patient was maintained on oral and intravenous carbohydrate solutions. The estimated average daily caloric intake during observation was 250 calories.

Autopsy findings. The body appeared well developed and moderately well nourished. The principal findings were marked acute renal tubular necrosis without evidence of pre-existing kidney disease, generalized peritonitis with early gangrene of the bowel, marked icterus and anemia.

The uterus, Fallopian tubes and ovaries were absent. The thyroid gland weighed 16.0 gm. The follicular epithelium was cuboidal and no histologic abnormalities were noted. The adrenal glands weighed 7.6 and 6.0 gm. The zona fasciculata had a slightly nodular configuration, but no significant lipoid depletion was noted. The parathyroid glands showed a prominence of large "water-clear" cells. The pancreatic islets were normal.

Case 2. A 75-year-old female developed symptoms of acute cholecystitis with marked nausea and vomiting 13 days before death. She was admitted to another hospital where her symptoms gradually subsided. Oliguria, however, was noted on the 4th day of illness, shortly followed by anuria. Her plasma non-protein nitrogen rose to 200 mg./100 ml. and the serum potassium to 7.0 m.Eq./L.

She was transferred to Emory University Hospital five days before death. She received a single hemodialysis the day before death, and one administration of insulin and intravenous glucose. She remained anuric. The terminal plasma non-protein nitrogen was 123 mg./100 ml., the serum potassium 7.5 m.Eq./L., and the blood hemoglobin 10.7 gm./100 ml. The temperature varied between 97.0° and 102.4°.

She was maintained on intravenous and some oral carbohydrates. The estimated average daily caloric intake during observation was 700 calories, but there was persistent nausea and vomiting.

Autopsy findings. The body was 160 cm. in length and weighed approximately 70 Kg. The principal findings were a subsiding acute and chronic cholecystitis, severe fatty metamorphosis of the liver and marked acute renal tubular necrosis.

The thyroid gland weighed 12.0 gm. There was minimal histologic nodularity and slight interstitial fibrosis, but the follicles were of normal size and the epithelium was generally cuboidal. The adrenal glands weighed 5.0 and 6.0 gm., had a normal architectural pattern, but showed severe lipoid depletion. The ovaries and endometrium were atrophic. The pancreatic insular tissue was histologically normal.

Case 3. A 37-year-old female developed vaginal bleeding at 18 weeks gestation. The fetal membranes ruptured and a macerated fetus was delivered at another hospital. The patient was in shock for 12 hours following delivery, despite several transfusions of plasma and whole blood. Persistent oliguria, 30-45 ml./day, developed the day after delivery (15 days before death). She was treated with intravenous glucose solutions, and several insulin injections. The blood urea nitrogen rose to 246 mg./100 ml., and she was transferred to Emory University Hospital. Past history revealed that she had been taking an unknown dose of a thyroid preparation for 15 months.

Hemodialysis was performed on the 5th and 3d days before death. Abdominal distention and paralytic ileus became severe despite gastric suction. The urinary output remained between 0 and 16 ml. per day. The temperature rose to 104° F. and icterus appeared. The terminal plasma nonprotein nitrogen was 153 mg./100 ml., the serum potassium 8.3 m.Eq./L., and the blood hemoglobin 12.2 gm./100 ml.

The estimated average caloric intake during observation was 700 calories per day. This consisted of intravenous and some oral carbohydrate solutions.

Autopsy findings. The body was well developed and well nourished. The principal findings were acute renal tubular necrosis, ruptured gravid uterus with acute endometritis and placenta accreta. Most of the placental tissue was necrotic but a rare viable villus was encountered. There was an acute peritonitis with 5,000 ml. hemoperitoneum and a central and midzonal hepatic necrosis.

The thyroid gland weighed 13.0 gm. The follicles were normal in size and were lined by cuboidal epithelium. A few small foci of fibrosis and lymphocytic infiltration were seen. The adrenal glands weighed 5.1 and 4.8 gm. and displayed severe lipoid depletion with an occasional focus of cortical cellular degeneration. The ovaries contained an involuting corpus luteum of pregnancy and normal ovarian stroma. Breast sections showed activ cretion. The islets of Langerhans were normal.

Case 4. This 29-year-old female was well until 6 days before admission to Emory University Hospital when she developed fever of 104° F., accompanied by chills, abdominal pain, nausea and vomiting. She was given parenteral penicillin, but developed a macular rash. The rash and fever subsided when tetracycline therapy was substituted. Persistent oliguria, however, was noted shortly after the onset of symptoms.

The plasma non-protein nitrogen was 200 mg./100 ml. The urine output during the first 7 days of hospitalization ranged from 50 to 315 ml./day, and the non-protein nitrogen rose to 365 mg./100 ml. The serum potassium remained within normal limits. Hemodialysis was performed on the 8th hospital day. The non-protein nitrogen fell to 181 mg./100 ml., and diuresis started. The urine output during the next 7 days ranged from 300 to 3,000 ml. per day. The non-protein nitrogen, however, rose again to 245 mg./100 ml. while the serum potassium fell to 2.4 m.Eq./L. The blood hemoglobin varied between 8.4 and 11.1 gm./100 ml. The temperature varied between 100° and 101° F. Nausea and vomiting became severe; there was increasing somnolence and death followed a generalized convulsion. The patient received only intravenous and oral glucose solutions after the second hospital day. The estimated average caloric intake during observation was 975 calories per day. Insulin and intravenous glucose were administered several times and she received 40 mg. ACTHAR gel daily during most of her hospital course.

Autopsy findings. The body was slender, measuring 162 cm. in length and weighing 52 Kg. The principal finding was an extensive renal tubular necrosis with a predominence of tubulorhexis. There was some central and midzonal hepatic necrosis, and focal myocardial necrosis compatible with the lesions of hypokalemia.

The thyroid gland weighed 12.5 gm. The follicles were normal in size and were lined by low cuboidal cells. The adrenal glands weighed 7.5 and 10.0 gm., and showed marked lipoid depletion. The ovaries were grossly and histologically normal, and the endometrium was in proliferative phase. The pancreatic islets were normal.

Case 5. This 68-year-old male underwent a transurethral prostatic resection 8 days before transfer to Emory University Hospital. He went into shock during the procedure and postoperatively developed persistent oliguria with a rise of blood urea nitrogen to 165 mg./100 ml., and serum potassium to 6.3 m.Eq./L.

He was treated with potassium adsorbing resin, intravenous glucose and insulin and fluid restriction. He remained afebrile. The urinary output averaged 170 ml./day, and the plasma non-protein nitrogen did not rise above 225 mg./100 ml. The blood hemoglobin fell from 10.2 to 7.6 gm./100 ml. He developed signs of congestive heart failure and died of acute pulmonary edema.

The estimated average caloric intake was 220 calories per day.

Autopsy findings. The body was moderately obese, measuring 175 cm. in length and weighing 76 Kg. The principal findings were acute renal tubular necrosis, acute pulmonary edema, and focal early bronchopneumonia.

The thyroid gland weighed 27.6 gm. There was slight interstitial fibrosis. The follicular epithelium varied between cuboidal and low cuboidal. The adrenal glands weighed 6.0 and 5.2 gm. There was marked lipoid depletion of the cortex and the zona glomerulosa appeared slightly nodular and thickened. The testes showed a marked decrease in spermatogenesis, and some decrease in Leydig cells. The pancreatic islets were normal.

Case 6. This 48-year-old female developed oliguria and then anuria following a subtotal gastrectomy for peptic ulcer. She became comatose the fourth day of illness and was transferred to Emory University Hospital. She had received only intravenous fluids.

The non-protein nitrogen at the time of admission to this hospital was 140 mg./100 ml., the serum potassium 4.9 m.Eq./L. She remained anuric and comatose. The temperature rose to 102° F., and she died on the 3d day of observation. Her caloric intake consisted entirely of intravenous glucose solutions, and averaged 500 calories per day.

Autopsy findings. The body appeared well developed, and well nourished measuring 167 cm. in length, and weighing approximately 70 Kg. The principal findings were acute renal tubular necrosis, and bilateral bronchopneumonia and pulmonary edema.

The thyroid gland weighed 15.0 gms. There was a diffuse interstitial fibrosis of moderate degree and some variation in the size of the follicles. Most of the follicles were lined by a low cuboidal epithelium. The adrenal glands weighed 6.0 and 7.0 gm. The architectural pattern was normal, but there was marked lipoid depletion. The ovaries appeared atrophic with no active germinal follicles, and the endometrium was atrophic. A parathyroid gland was histologically normal with no evidence of hyperplasia. The islets of Langerhans were unremarkable.

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J. C. OPSAHL*
K. KOWALEWSKI**

Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Department of Physiology, Yale University School of Medicine, and the McEachern Cancer Research Laboratory, University of Alberta, Canada

FURTHER STUDIES ON PLACENTAL ACTH, PITUITARY, AND ADRENAL-HYALURONIDASE RELATIONS: The Influence of Placental ACTH on the Excretion of Urinary Corticoids and 17-Ketosteroids in Guinea Pigs†

During the past several years, an investigation has been made of the relationships between various hormones of the adrenal cortex, the enzyme hyaluronidase, and its substrate hyaluronic acid, with specific reference to tissue permeability and collagen diseases. As the studies progressed, several problems were investigated: (i) which fractions of the adrenal cortex were responsible for the results obtained, (ii) what mechanism or mechanisms of action were involved, (iii) in what way the placenta, as well as the pituitary gland, was involved in hyaluronic acid metabolism in relation to connective tissue disease processes. In other studies, experimental evidence has suggested an action of these adrenal hormones on the enzyme hyaluronidase *in vitro*,[10] while desoxycorticosterone, growth hormone, and the related sex steroids, are without hyaluronidase-inhibitory effect.

Adrenocorticotrophic hormone was demonstrated to be a potent inhibitor of the hyaluronidase-enhanced spreading phenomenon through the release of the effective C-11 adrenal steroids in the intact rabbit, rat, guinea pig, mouse, and dog.[12]

In view of the favorable influence of pregnancy on certain of the collagen diseases, it was of interest to demonstrate that chorionic gonadotrophin obtained from human pregnancy urine exhibited marked hyaluronidase-inhibitory activity in normal, castrate, and hypophysectomized animals of both

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* Research Associate in Medicine, Harvard Medical School; Assistant in Medicine, Peter Bent Brigham Hospital; Physician, PBBH School of Nursing.

** Assistant Professor, Department of Experimental Surgery, University of Alberta, Edmonton, Alberta, Canada.

† This work was supported by the Office of Naval Research, Department of the Navy, Contract Nonr: 1866(22), and by the Damon Runyon Cancer Foundation.

Received for publication 28 October 1959.

sexes in the presence of the adrenals, but was entirely without effect in adrenalectomized animals. This inhibition also was produced by chorionic gonadotropin preparations that had been heat inactivated to destroy gonadotrophic activity, indicating that the effect was not due to the gonad-stimulating principle but to a fraction that exhibited many of the properties previously associated with ACTH from the pituitary tissue.[13]

These experimental findings led to investigations of placental tissue to ascertain whether the source of the ACTH activity was the placenta itself rather than the pituitary. Preliminary investigations of placental extracts, using inhibition of exogenously added hyaluronidase as an index of adrenal activity, indicated that significant quantities of ACTH are produced by the human placenta although the crude methods of isolation employed did not permit quantitative interpretations of yields or of purity. The hyaluronidase-inhibitory effects produced by the various placental fractions were markedly similar to those produced by the heat-inactivated human chorionic gonadotropin preparations. It seemed, therefore, quite appropriate to speak of placental ACTH as a substance that causes the adrenal to produce a factor or factors that inhibit hyaluronidase, but not necessarily to assume placental ACTH to have physiological characteristics identical with those of pituitary ACTH.

That the placenta influences in some way the excretion of certain urinary steroids may be presumed from the work of Venning and Browne[18] who noted the increase of urinary corticoids in the last trimester of pregnancy. It was, therefore, of interest to establish whether a urinary steroid pattern similar to that observed following administration of pituitary ACTH would result from administration of placental ACTH to normal and hypophysectomized animals. Forsham *et al.*[5] have shown that pituitary ACTH causes significant rises in the excretion of both corticoids and 17-ketosteroids in man. Burstein[3] observed a rise of 400 per cent in the excretion of corticoids in guinea pigs, after the injection of 1 mg. of pituitary ACTH per animal. An increase of excretion of 17-ketosteroids in guinea pigs treated with ACTH was reported by Clayton and Prunty.[3] The increased excretion of 17-ketosteroids in normal and hypophysectomized rats after injection of ACTH was noted by Kowaleski.[6] We have studied the influence of crude placental ACTH fractions on the excretion of urinary corticoids and 17-ketosteroids in guinea pigs and in rabbits. Results from investigations point out that the measurement of urinary steroid excretion in guinea pigs is a reliable method for the evaluation of ACTH activity in placental tissues, and that this method may be used as a further assay procedure.

MATERIALS AND METHODS

It has been experimentally established that the choice of suitable animals for the study of urinary steroids is of some importance.* Burstein[8] demonstrated that guinea pigs are sensitive and reliable animals for the determination of urinary corticoids, and Clayton and Prunty[8] have reported comparable results from their studies of urinary 17-ketosteroid excretions in guinea pigs.

Forty-two male guinea pigs from our own colony, fed on commercial rabbit pellets and water *ad libitum*, were employed. The weights of animals ranged from 425 to 675 grams. During the experimental period, and for 10 days preceding the collection of urines, no carrots nor other colored vegetables were given. Urines were collected over a 17-hour period from fasting animals kept in separate metabolic cages, as suggested by Burstein.[8] Animals were fed and received water during a 7-hour interval when the collection of urines was discontinued. The injection of test substances was done immediately before placing the animals in the cages for urine collections.

Acidified-acetone preparations of crude placental ACTH prepared by our modification of the Lyon's procedure for the extraction of mammotropic hormone, previously described in detail,[14] were used in these experiments. These preparations were dissolved in sterile 0.9 per cent saline (10 mg. per cc.) and were injected intraperitoneally or subcutaneously in amounts of 20 mg. per kilogram of body weight.

Three control series of animals were employed in these studies. One group received pituitary ACTH (Nordic Biochemicals Ltd.) in doses of 2 mg. per kilogram of body weight. Another group was treated with an extract of liver tissue prepared by the same procedure and given in the same doses as the placental ACTH fractions. The third group received saline in amounts of 2 ml. per kilogram of body weight. In all cases, 2 ml. of solution per kilogram of body weight of animals were given.

The phosphomolybdic acid method, as described by Heard and Sobel,[6] was used for the determination of corticoids. Phosphomolybdic acid was prepared, omitting the tungstic acid as suggested by Burstein,[8] and the extraction and evaporation of extracts was carried out. As shown from the study of corticoids in human urine by Romanoff[15] and from published experimental results by Burstein on corticoid excretion in guinea pigs, the phosphomolybdic method permits the evalution of ACTH activity with the same precision as does the formaldehydrogenic method.

The 17-ketosteroids were determined with the modification of classic techniques as described by Masuda and Thuline.[9] In this modification, the Zimmermann color is extracted with amylacetate which leaves behind, in the aqueous layer, the nonketonic interfering chromogens, as was proved by the micro-Girard separation and chromatography.

Only male guinea pigs were used in these studies. As noted by Zondek and Burstein,[19] there is a cyclical variation of both corticoids and 17-ketosteroids in the urine. These results were also noted in women by Venning and Kazmin.[17] We therefore used male animals, in which existing daily variations are acyclical.

* Guinea pigs have the largest adrenal-weight/body-weight ratio among animals thus far studied; the ratio being 0.1 for guinea pigs, and 0.01 for adult man. This difference would serve to explain why values for urinary steroids are relatively high in guinea pigs.

The methods and precautions as to general procedures, and test substances studied, as well as the extraction procedures employed, have been described in detail in our earlier publications.

EXPERIMENTAL RESULTS

The noted daily variations of resting values of the excretion of both corticoids and 17-ketosteroids in guinea pigs are in accord with the results of

TABLE 1. INFLUENCE OF PLACENTAL AND PITUITARY ACTH ON THE URINARY EXCRETION OF CORTICOIDS (MICROGRAMS/17 HRS.) IN MALE GUINEA PIGS

Treatment:		*None*				*Placental ACTH*	*None*		*Pituitary ACTH*		*None*	
Days:		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*		*9*	*10*
Animals	*B.W. Gm.*											
1	425	231	181	160	242	668	231	184	506		268	277
2	560	121	210	200	206	744	276	252	521		329	300
3	600	161	210	181	184	878	259	230	667		147	182
4	650	131	260	260	224	878	182	200	582		276	277
5	560	128	130	230	247	705	182	182	490		256	240
6	650	231	130	230	247	520	188	190	537		153	166
	Days:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
7	490	170	181	173	187	500	216	238	646	542	168	193
8	495	200	218	200	178	528	246	266	868	694	271	207
9	530	219	140	182	200	544	266	268	640	604	174	201
10	588	282	208	173	196	652	184	246	668	656	295	220
11	605	229	256	191	178	636	192	206	650	674	271	297
12	612	200	236	182	212	652	274	160	620	638	300	242

Dosage: Placental ACTH: 20 mg. (2 ml.)/Kg. B.W. intraperitoneally.
Pituitary ACTH: 2 mg. (2 ml.)/Kg. B.W. intraperitoneally in animals 1-6.
Pituitary ACTH: 2 mg. (2 ml.)/Kg. B.W. subcutaneously in animals 7-12.

Burstein[2] and of Clayton and Prunty[3] who studied urinary steroid excretion in animals, and of Kowaleski[7] who presented comparable results from studies in man. This variability necessitated repeated determinations of steroid excretion during stated time intervals preceding the administration of test substances. The results of our experiments are presented in the following tables:

Table 1 indicates the values of corticoid excretion in 12 guinea pigs before and after the injection of placental and pituitary ACTH. Placental ACTH

was injected intraperitoneally into all animals; pituitary ACTH was given intraperitoneally to six guinea pigs, and subcutaneously to a second group of six guinea pigs. Subcutaneous injection was made during the two 17-hour periods. The range of resting basic values for 12 animals varied from 121 to 282 μgm. The range of values following a single intraperitoneal injection of placental ACTH, was from 500 to 878 μgm. The marked rise of corticoid excretion following administration of placental ACTH was of

TABLE 2. URINARY EXCRETION OF CORTICOIDS (MICROGRAMS/17 HRS.) IN MALE GUINEA PIGS AFTER INJECTION OF NORMAL SALINE AND PLACENTAL ACTH

Treatment:		*None*		*Saline*	*None*	*Placental ACTH*		*None*	
Days:		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Animals	*B.W. Gm.*								
13	464	193	218	238	193	523	554	206	209
14	490	212	206	200	244	575	578	182	190
15	487	212	245	238	251	591	594	215	185
16	520	231	252	193	180	480	487	188	212
17	465	171	182	200	170	512	501	212	179
18	531	238	245	192	226	543	563	209	190
19	510	213	231	238	245	560	532	181	197
20	496	182	193	182	231	512	498	189	197

Dosage: Saline: 2 ml./kg. B.W. intraperitoneally.
Placental ACTH: 20 mg. (2 ml.)/kg. B.W. subcutaneously.

short duration and the following 17-hour period resulted in the return of corticoid excretion to nearly resting values (ranging from 182 to 276 μgm). Comparable results were observed after the injection of pituitary ACTH, and no notable differences were obtained between the groups injected subcutaneously or intraperitoneally.

Table 2 shows the results of intraperitoneal injection of saline (2 cc. per kilogram of body weight) on the excretion of corticoids, and the effect of placental ACTH injected subcutaneously on corticoid excretion. These studies were made to exclude the possibility of non-specificity or trauma as a factor in the results observed, since it has been pointed out by Forbes[4] and by Talbot[16] that trauma may effect urinary steroid excretion. As may be seen from the results of these studies, no notable influence was observed following the injection of saline. Table 2 shows, also, that the subcutaneous administration of placental ACTH provoked a definite rise in the excretion

of corticoids during the period of treatment. Comparison of data in Tables 1 and 2 would indicate that the intraperitoneal injection resulted in slightly higher values than the subcutaneous one, but in both cases, the results of adrenal trophic activity of placental ACTH were self-evident.

Table 3 represents the results of other control experiments. A liver extract was administered to 10 guinea pigs, intraperitoneally, in doses of 20 mg. per kilogram of body weight. Urinary corticoids were examined before, during and after treatment. No significant differences in corticoid excretion were noted as a result of this treatment.

TABLE 3. EXCRETION OF URINARY CORTICOIDS (MICROGRAMS/17 HRS.) AFTER THE INJECTION OF LIVER EXTRACT

Treatment:		*None*			*Liver tissue extract*		*None*	
	Days:	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Animals	*B.W. Gm.*							
21	644	120	173	148	210	230	211	183
22	650	146	132	174	160	205	194	172
23	700	205	261	246	252	245	250	260
24	702	194	170	234	242	260	243	231
25	720	230	210	225	240	190	232	194
26	750	219	192	240	203	244	218	188
27	770	241	210	270	242	261	254	236
28	772	214	272	280	264	282	270	244
29	780	277	280	245	286	255	250	271
30	895	261	310	267	292	259	248	258

Dosage: 20 mg. (2 ml.)/kg. W.W. intraperitoneally.

Table 4 presents the values of 17-ketosteroid excretion in guinea pigs when placental ACTH was injected intraperitoneally, and pituitary ACTH was injected subcutaneously. The range of basic vales for 12 animals examined during the two 17-hour periods prior to treatment was from 200 to 300 μgm (24 determinations). The placental ACTH, injected the third day, resulted in a definite rise in the excretion of 17-ketosteroids, the range of values after treatment being from 615 to 928 μgm. The injection of pituitary ACTH also resulted in a definite increase of urinary 17-ketosteroid excretion in the animals treated[17-19] during the 17-hour periods, and when treatment was discontinued, the return to nearly basic values was observed.

Table 5 summarizes the results presented in Tables 1-4. Review of the tables indicates that the results are statistically significant. There is no overlapping of values of steroid excretion found before and after treatment in the various groups of animals. High standard deviations noted in a few groups of treated animals are due to the large range of values of steroids found after the injection of tested substances.

TABLE 4. THE INFLUENCE OF PLACENTAL AND PITUITARY ACTH ON THE URINARY EXCRETION OF 17-KETOSTEROIDS (MICROGRAMS/17 HRS.) IN MALE GUINEA PIGS

Treatment:		*None*		*Placental ACTH*	*None*		*Pituitary ACTH*		*None*	
	Days:	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Animals	*B.W. Gm.*									
31	540	220	283	690	410	192	...	...	...	...
32	490	340	262	928	234	210	...	...	...	...
33	496	246	282	740	270	232	...	...	...	...
34	580	364	316	892	394	376	...	...	...	...
35	690	288	309	786	588	382	...	...	...	...
36	675	323	268	668	240	188	...	...	...	...
37	610	200	312	735	278	245	780	564	337	255
38	645	230	312	635	270	276	855	784	281	281
39	615	296	234	775	215	212	855	816	318	289
40	605	278	234	635	226	238	880	656	327	271
41	618	278	296	690	253	244	830	764	255	261
42	646	242	270	615	235	226	910	880	289	265

Dosage: Placental ACTH: 20 mg. (2 ml.)/Kg. B.W. intraperitoneally.
Pituitary ACTH: 2 mg. (2 ml.)/Kg. B.W. intraperitoneally.

DISCUSSION

The methods employed in the investigation of ACTH and adrenal steroid activity have shown wide variation in qualitative and quantitative response to materials of varied physiologic origin. This variation has also reflected the state and method of purification or extraction, dosage regimen, and route of administration. These data indicate that, despite daily variations in urinary steroid excretion, such assays may serve as valid indices of adrenal cortical activation. These data further tend to confirm material presented in an earlier paper[14] concerning the presence of ACTH in human placental tissues and to indicate that measurements of urinary steroid excretion are reliable for the evaluation of placental ACTH activity and for

confirming of purification procedures for this substance in addition to hyaluronidase-inhibition studies hitherto performed.

The data depicted in Figure 1 demonstrate that crude placental ACTH, extracted by acidified-acetone fractionation procedures,[14] produces a marked increase in urinary excretion of corticoids and 17-ketosteroids entirely comparable with the results produced by the administration of pituitary

TABLE 5. AVERAGE VALUES, STANDARD DEVIATIONS, AND THE RANGES OF EXCRETION OF URINARY STEROIDS IN MALE GUINEA PIGS; SUMMARIZING RESULTS PRESENTED IN TABLES 1-4

Treatment:	*Mode of injection*	*No. of animals*	*Corticoids, (micrograms/17 hrs.)* *Day before injection:*	*Day after injection:*
Placental ACTH	Intraperitoneally	12	208 ± 25 (178-247)	659 ± 123 (500-878)
	Subcutaneously	8	217 ± 29 (170-251)	537 ± 36 (480-591)
Saline	Intraperitoneally	8	221 ± 24 (182-252)	210 ± 19 (182-238)
Liver tissue extract	Intraperitoneally	10	243 ± 41 (148-280)	239 ± 38 (160-292)
Pituitary ACTH	Intraperitoneally	6	204 ± 26 (182-252)	550 ± 36 (490-667)
	Subcutaneously	6	230 ± 37 (160-268)	682 ± 84 (620-868)
			17-Ketosteroids, (micrograms/17 hrs.)	
Placental ACTH	Intraperitoneally	12	281 ± 28 (234-316)	732 ± 95 (615-928)
Pituitary ACTH	Intraperitoneally	6	270 ± 34 (212-276)	851 ± 50 (780-910)

ACTH. This was found when the intraperitoneal or subcutaneous route was employed. The injection of saline and liver extract is shown to produce no significant alteration ascribable to non-specificity or trauma.

While the assay of hormonal materials by inhibition of exogenously added hyaluronidase in hyaluronidase-enhanced spreading (hypophysectomized mice or rats) indicates that acetic acid-extracted pituitary and placental ACTH have roughly the same order of activity, alteration of the extraction procedure produces variation in results (Fig. 2) indicated as diminution of the activity of purified materials.

When other assay techniques such as the mobilization of adrenal ascorbic acid or eosinopenic response are employed in investigations of the activity of placental or pituitary preparations, wide variations in activity are noted.

These variations may be dependent upon dosage administered, experimental animals employed, and routes or times of administration used in the study of test preparations. In these assays, purification procedures induce further variations reflected as *increased* activity of the purified materials.

The identity of results obtained when the urinary steriod excretion level is employed as the assay procedure, despite the use of materials of varied physiologic origin, or route of administration, recommends its employment

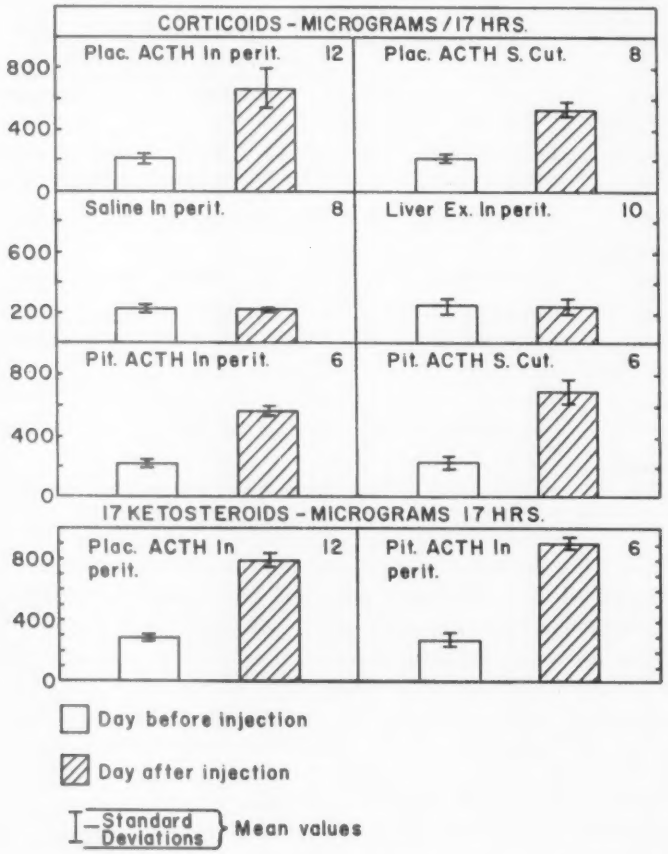

FIG. 1. Comparison of placental and pituitary ACTH on excretion of urinary steroids in male guinea pigs.

as a further index of adrenal cortical activation and, in addition, confirms our earlier work concerning the presence of ACTH in human placental tissues.

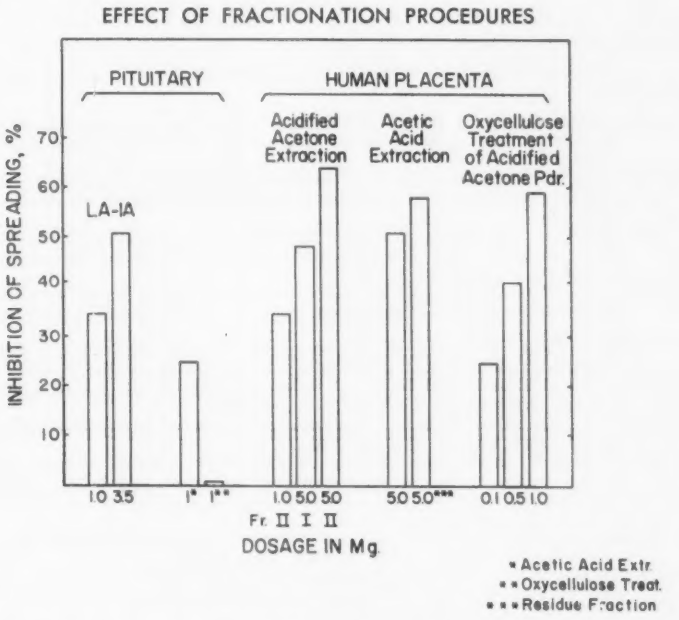

FIG. 2. Inhibition of hyaluronidase-enhanced spreading reaction in hypophysectomized mice (placental and pituitary ACTH).

SUMMARY

1. Further confirmation of our earlier work concerning the production of ACTH by the human placenta is presented.

2. The administration of crude placental ACTH results in a marked increase in the urinary excretion of 11-oxycorticoids and 17-ketosteroids in male guinea pigs.

3. Recent work from our laboratories has led to the conclusion that stimulation of the adrenal cortex by ACTH of either pituitary or placental origin results in a physiological mixture of hormones that is more effective than would be predicted on the basis of individual components, or, in the production of hyaluronidase-inhibitory hormones not thus far studied.

4. Further suggested is the possibility that placental ACTH of human origin may well be obtained for clinical usage.

ACKNOWLEDGMENT

Appreciation is expressed for the statistical analysis of the data presented herein by Mr. Neil Stasilli of the Warner-Lambert Research Institute, Morris Plains, New Jersey.

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LAWRENCE R. FREEDMAN*
EDVARDAS KAMINSKAS**
PAUL B. BEESON†

Department of Internal Medicine, Yale University School of Medicine

EXPERIMENTAL PYELONEPHRITIS. VII. EVIDENCE ON THE MECHANISMS BY WHICH OBSTRUCTION OF URINE FLOW ENHANCES SUSCEPTIBILITY TO PYELONEPHRITIS‡

One of the key problems in the pathogenesis of pyelonephritis is to explain how obstruction of urine flow renders the kidney more susceptible to infection. Since urine is a good culture medium, slowing of its excretion doubtless provides opportunity for more luxuriant bacterial multiplication, but there are indications that other consequences of urinary obstruction favor the growth and survival of bacteria in the tissues of the kidney. The purpose of the present communication is to review the existing evidence on this subject, and to report some additional pertinent experiments. The latter include observations on the development of acute pyelonephritis when the urine contained a concentration of antibiotic sufficient to inhibit growth of bacteria, and a comparison of the relative degree of susceptibility conferred by obstructive lesions of different ages.

MATERIALS AND METHODS

The techniques utilized have been described in previous reports from this laboratory.[5, 7, 9] All studies were performed with white New Zealand male rabbits weighing 2-3 Kg.

RESULTS

Renal infection by a streptomycin-sensitive organism during excretion of streptomycin in the urine. Utilizing the fact that streptomycin is concentrated during excretion in the urine, experiments were designed to test whether pyelonephritis could develop at a time when the urine contained an inhibitory concentration of this drug. Preliminary tests showed that an

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* Markle Scholar in Medical Science; Assistant Professor of Medicine.
** Intern, Michael Reese Hospital, Chicago, Illinois.
† Ensign Professor of Medicine.
‡ A portion of this work was submitted by one of the authors (E.K.) in partial fulfillment of the thesis requirement for the M.D. degree, Yale University School of Medicine, 1959.
Aided by a research grant from the U.S. Public Health Service (E-1850).
Received for publication 25 January 1960.

effective antibiotic level could be expected for several hours after the intramuscular administration of 5 mg.

Six rabbits were employed in these experiments. Each received a preliminary intramuscular injection of streptomycin. One hour later the animal was anesthetized, the peritoneal cavity opened, bladder urine was aspirated for test of its antibacterial properties, and then a second intramuscular injection of streptomycin was given. To induce pyelonephritis, 0.05 mg. of diluted bacterial culture, containing 10^6 to 10^8 viable *E. coli,* was injected into the medullary tissue of the kidney. As described previously,[8] this procedure causes the prompt escape of some bacteria into the urine, and the development of acute suppurative infection in the segment of kidney obstructed by the needle puncture. The present experiments were each terminated at the end of six hours following the bacterial inoculation. At that time the bacterial contents of whole kidney and of bladder urine were determined. In two of the six animals growth was not obtained from kidney tissue nor from bladder urine, undoubtedly because of the presence of an inhibitory concentration of streptomycin in both areas. In the remaining four, however, although bladder urine cultures were sterile, there was evidence of active bacterial multiplication in the tissues of the kidneys, since the number of organisms found there was 10^6 to 10^7. Tests of the bladder urines of all six animals showed that at the time of bacterial inoculation and at the time of conclusion of the experiments the concentration of antibiotic was sufficient to prevent growth of the test organism *in vitro.*

As discussed in the original description of this form of experimental pyelonephritis, the needle puncture through which the bacteria are delivered to the medulla produces a zone of intrarenal obstruction. The resulting area of infection remains confined to the zone of medulla and cortex affected by this small obstructive lesion. A question which must be considered, therefore, is whether one can assume that the streptomycin effect was the same in the obstructed tubular fluid as in the bladder urine. To gain information on this point the same experiment was carried out in five rabbits with the single modification that the ureter was ligated just before bacteria were injected into the medulla. This would obstruct all nephrons, including those whose collecting ducts had been severed by the needle puncture. Despite the extreme susceptibility which is characteristic of the totally obstructed kidney, culture six hours later gave no bacterial growth in any animal, indicating that streptomycin was highly effective in the presence of obstruction of all nephrons. The fact that it prevented bacterial survival in the kidney tissue is probably attributable to diffusion from the trapped urine throughout all tissues. At any rate it seems justifiable to assume that

the degree of intrarenal obstruction caused by the needle puncture did not nullify the effect of streptomycin already present in the obstructed nephrons.

Duration of obstructing lesion in relation to susceptibility to infection. Obstructing lesions of different ages were compared with respect to their influence on susceptibility to infection. The type of obstruction was a small medullary injury produced by a surgical cautery. Intravenous inoculation of 10^8 *E. coli* after an interval of one week results in acute pyelonephritis in about 85 per cent of instances.[9] In the present study 23 rabbits were subjected to this type of obstructive renal lesion, but the bacterial challenge was delayed for two to four months. On subsequent examination, one week after the hematogenous infection, no gross infections were found, and cultures of blood, urine and kidneys gave no growth, save for a single kidney which contained 10^8 bacteria. The injured kidneys all showed medullary scars and dilatation of the nephrons tributary to the regions of the scars. As a control on the infectivity of the test strain of bacteria, six additional animals were given the same renal injury and then challenged by intravenous inoculation after an interval of only one week. Abscesses resulted in five of these kidneys, and cultures revealed the presence of more than 10^8 bacteria.

It appears, then, that a kidney with medullary obstruction and intrarenal hydronephrosis is more susceptible to infection one week after injury than after two to four months. Obviously there are great diifferences in these lesions at the two time intervals. We would emphasize, however, that there was pronounced tubular dilatation in considerable sections of the kidneys in all preparations, and that the only point under scrutiny here is whether growth of bacteria in stagnant urine is the principal factor in the susceptibility to infection which characterizes obstructive lesions.

DISCUSSION

The experiments reported in this article were discussed as they were presented. Neither type can be regarded as offering conclusive proof, but the findings seem to indicate that obstruction affects the susceptibility of the kidney to infection by some means other than the provision of a stagnant pool of culture medium from which bacteria invade renal tissue. The findings are in keeping with the concept that the host-parasite relationship is affected by obstruction in such a way that bacteria flourish in the renal tissues, and then spread to the urine. The salutary clinical responses which commonly result from relief of obstructive lesions are therefore probably attributable to more than the mere speeding of urine flow.

There is no intent here to minimize the importance of urine as an excellent culture medium, scarcely affected by the principal host mechanisms of antibacterial action, and serving as a reservoir for bacteria as well as a vehicle for their transport between different parts of the urinary tract. A large volume of clinical observation suggests that the commonest sequence of events in the development of human pyelonephritis is first the colonization of bladder urine by bacteria, then, and especially in the presence of obstruction, the occurrence of pyelonephritis.[1] Animal experiments have given support to the concept of 'ascending infection.' For example, it was shown in this laboratory that pyelonephritis could be produced by inoculating *E. coli* into the bladder urine of a rabbit with renal medullary injury.[8] More recently Vivaldi *et al.* have reported that pyelonephritis developed in rats even in the absence of renal injury, when a strain of Proteus bacteria was inoculated into the bladder cavity.[11]

It is evident that microorganisms can make the trip from bladder to kidney without difficulty in the normal animal. With relatively virulent organisms such as Proteus or enterococcus, delivery of a sufficient inoculum is the critical prerequisite for infection. However, in the case of coliform bacteria, which are the principal causes of human pyelonephritis, some additional factor is generally required for the occurrence of renal infection.

The hypothesis that obstruction of urine flow facilitates bacterial growth in the renal tissues is supported by the following pieces of evidence, obtained in animal experiments:

1. Organisms capable of causing hematogenous pyelonephritis in normal animals, such as staphylococcus and enterococcus, could not be demonstrated in the urine until after bacterial multiplication was demonstrable within the kidney.[6,9]

2. Pyelonephritis was induced by ligating the ureter three or four days after intravenous injection of bacteria, at a time when only a few living organisms were present in the kidney and none was being excreted in the urine.[7] It is difficult to avoid the conclusion that ligating the ureter created some effect which enabled the few bacteria lying in the kidney tissue to multiply and massively infect the entire kidney.

3. The effect of obstruction on the kidney's susceptibility to infection seemed clearly evident in the pattern of distribution of the acute infectious process which resulted from medullary injury and inoculation via the blood stream. This lesion was described as follows: " . . . one of the most impressive findings was the observation that acute pyogenic infection tended to remain confined to the area of the kidney affected by the medullary lesion, in spite of the fact that the urine in the pelvis of the kidney teemed with bacteria. In the acute stage, at any rate, there seems to be little tendency for unobstructed portions of the kidney to become infected, either by way of lateral spread in the kidney substance or from the pelvic urine."[9]

4. Ureteral ligation, although causing stagnation of urine flow throughout the length of the nephrons, did not markedly augment the susceptibility of renal *cortical* tissue. Even in the presence of total renal obstruction it was possible to demonstrate a marked difference in infectivity of the renal cortex and the medulla.[8] This points to some determinant of renal susceptibility other than the availability of a pool of tubular fluid.

5. When hydronephrosis was produced in rats and rabbits by *gradual* ureteral constriction only a slight increase in susceptibility to infection resulted, in contrast to the effect of *sudden* occlusion of the ureter of a previously normal kidney.[8] This again seems to indicate that stagnation of urine flow is not the principal factor in the initiation of acute pyelonephritis.

It would appear that obstruction alters renal resistance to infection not only by making available a stationary supply of culture medium, but probably also by transmitting increased pressure to the kidney parenchyma. This may be the explanation for the observation by Brumfitt and Heptinstall, that obstruction of the renal vein leads to an increased susceptibility to hematogenous pyelonephritis,[9] since Gottschalk has shown that the pressure effects of ureteral obstruction can be duplicated by increasing renal venous pressure.[6]

The precise means by which increased tissue pressure lowers resistance to infection cannot be stated. It has been shown, however, by anatomical and functional studies that there is a diminution of renal blood flow associated with ureteral obstruction.[10] This may cause alterations in lymph flow, and could affect the function of humoral or cellular host defense mechanisms. Whatever the nature of this change the effect is less easily demonstrated when the obstruction is gradually produced and when it has been present for some months.

SUMMARY

Experiments were designed to study further the mechanism by which obstruction of urine flow increases the susceptibility of the kidney to infection, with particular reference to the factor of bacterial growth in the stagnant fluid. In animals treated with small doses of streptomycin, bacteria multiplied readily in an obstructed segment of the kidney when they could not survive in the urine. It was found also that hematogenous pyelonephritis occurred more readily in association with recent than with old obstructive lesions. These results suggest that the presence of stagnant tubular fluid is not the only factor which accounts for the effect of obstruction.

The evidence presented, together with other observations, is taken to indicate that urinary obstruction lowers the resistance of renal tissue to infection because of some secondary effect of increased tissue pressure.

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OLGA STEIN*
JOSEPH S. FRUTON**

Department of Biochemistry, Yale University School of Medicine

PROTEINASE ACTIVITY OF RAT LYMPH NODES†

INTRODUCTION

Proteolytic activity in extracts of lymphatic tissue has been described in the older literature,[11] gelatin, casein, and serum albumin having been used as substrates. More recently bovine tonsils were reported to contain a protease that cleaves serum albumin.[7] Rothschild and Junqueira[13] found proteolytic activity in lymph node homogenates, using globin as substrate.

In the present investigation, an attempt was made to characterize the proteolytic activity of rat lymph nodes by using both synthetic and protein substrates, and to relate the proteolytic activity of this tissue to that previously described for enzymes derived from bovine spleen.[4, 6, 9]

MATERIALS AND METHODS

Mesenteric and cervical lymph nodes from Sprague-Dawley rats of both sexes were used in all experiments. The animals were sacrificed by cervical severance and the lymph nodes were quickly excised, cleaned of all surrounding fat, and homogenized for 20 seconds in an all-glass Potter-Elvehjem homogenizer with 3 volumes of 0.25 Msucrose—0.001 M Versene. All operations were carried out in the cold (0°-5° C.). The Spinco model L preparative ultracentrifuge (R 30 and R 40 rotors) was employed in the preparation of subcellular fractions, and the following procedure was used. The homogenate was centrifuged for 5 minutes at 2,100xg; the sediment was then resuspended and rehomogenized for 20 seconds with sucrose-Versene, and centrifuged at 600xg for 10 minutes. For the preparation of nuclei this sediment was resuspended, washed, and rehomogenized twice. The supernatant fluid from the 600xg and the 2,100xg centrifugations was combined and spun at 20,000xg for 18 minutes. The fluffy layer that formed on top of the tightly packed sediment was included in the supernatant fluid (designated Sup I). Several preparations of Sup I were lyophilized and used as the source of enzyme. The sediment was resuspended by gentle homogenization and respun at 20,000xg for 13 minutes. The resulting sediment (designated the mitochondrial fraction, Mit) was then resuspended in a small volume

* Research Associate in Biochemistry. Permanent address: Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

** Eugene Higgins Professor of Biochemistry.

† This investigation was aided by grants from the National Science Foundation (G-7451) and the U.S. Public Health Service (RG-6452).

Received for publication 5 April 1960.

of sucrose. The microsomal fraction (Mic) was prepared by centrifuging Sup I for 30 minutes at 105,000xg and the resulting supernatant fluid was called Sup II. In some experiments the mitochondrial fraction was further fractionated; the heavier material (H. Mit) was sedimented from the post-nuclear supernatant fluid by two centrifugations at 6,600xg for 5 minutes each, while the lighter material (L. Mit) was sedimented at 20,000xg for 13 minutes, followed by 3 washings. When no separation of the microsomal and mitochondrial fractions was attempted, the material sedimenting at 105,000xg (after the removal of nuclei) was prepared and designated "particles." The protein content of each fraction was determined by the biuret method.[10]

The synthetic substrates used were benzoyl-L-argininamide · HCl · H_2O (BAA) and glycyl-L-tyrosinamide acetate (GTAA). Unless otherwise stated, the incubation

TABLE 1. PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARDS BENZOYL-L-ARGININAMIDE

Incubation volume, 0.7 ml.; pH 5.7; enzyme preparation, "particles."

	NH_3 released per mg. protein	
Protein mg.	*90 min. per cent*	*180 min. per cent*
0.5	8.4	16.0
1.0	8.4	16.0
2.0	8.3	15.6
2.7	10.0	15.0

mixture (0.6 or 0.7 ml.) contained 0.04 M substrate, 0.015 M cysteine, 1 per cent Triton X-100 (Rohm and Haas), and a subcellular fraction of the lymph node preparation. The pH was maintained with 0.1 M citrate buffer in the region of pH 3 to 6, or with 0.2 M phosphate buffer above pH 6. All incubations were carried out at 38° C. To determine the proteolytic activity of the subcellular fractions towards BAA and GTAA the Conway microdiffusion method was employed for the determination of ammonia liberated upon hydrolysis of the amide bond.[8] The enzymic action is expressed as per cent NH_3 released in a stated time interval; specific activity (S.A.) refers to per cent cleavage of the substrate/mg. protein/hour.

To determine the extent of transamidation in the enzyme-catalyzed reaction between benzoyl-L-argininamide and hydroxylamine, the hydroxamic acid formed was determined according to the procedure described before.[8]

The proteins used as substrates were horse hemoglobin and bovine serum albumin (Pentex). With hemoglobin as substrate, a modification of the method of Anson as described by Tallan *et al.*[18] was employed. With serum albumin a similar spectrophotometric procedure was followed. Unless otherwise stated, the incubation mixture (0.65 ml.) contained: 4 mg. of hemoglobin or 10 mg. of serum albumin, 2.5 mg. protein of the appropriate subcellular fraction and 0.03 M cysteine. The pH was maintained with 0.1 M citrate buffer at pH 3.5 and 5.0 and with 0.2 M tris (hydroxymethyl) aminomethane buffer at pH 7.2. After 120 minutes of incubation at 38° C. the reaction was stopped by the addition of 2.5 ml. of 10 per cent trichloroacetic acid (TCA), and the volume was made up to 5 ml. with water. The optical density of the

TCA supernatant fluid was determined in the Beckman spectrophotometer (1 cm. cells) at 280 mμ, with distilled water as the blank. Enzyme, substrate and reagent blanks were run in parallel. Proteinase activity toward both hemoglobin and serum albumin is expressed in terms of the number of cathepsin units $[C.U.]^{Hb}$ and $[C.U.]^{Alb}$ respectively. One cathepsin unit is defined as that amount of enzyme, which in the 0.65 ml. of test solution (diluted to 5 ml. after TCA precipitation), in 120 minutes of incubation and under the conditions specified above, causes an increase in optical density of 0.100 (after appropriate correction for the blanks).

TABLE 2. SUBCELLULAR DISTRIBUTION OF PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD BENZOYL-L-ARGININAMIDE AND GLYCYL-L-TYROSINAMIDE

Incubation volume, 0.6 ml.; pH 5.7; time, 180 min.

	NH_3 released		*Specific activity**	
Fraction	*BAA per cent*	*GTAA per cent*	*BAA*	*GTAA*
Nuclear	16.5	21.0	1.8	2.3
Mitochondrial	65.0	89.0	8.3	15.0
H. Mitochondrial	17.0**		4.2	
L. Mitochondrial	46.5**		9.1	
Microsomal	10.6**		1.8	
Sup II	5.8	11.8	1.6	3.3

* Per cent hydrolysis/mg. protein/hour.
** Time, 120 min.

RESULTS

As shown in Table 1, BAA is split by the particles prepared from rat lymph node homogenates; within the range 0.5-2.0 mg. protein, the enzymic activity is proportional to the protein concentration and the time of incubation.

The subcellular distribution of the enzymic activity toward BAA and GTAA was investigated using a differential centrifugation scheme similar to that of de Duve *et al.*[1] It can be seen in Table 2 that the highest specific activity (per cent cleavage of substrate per mg. of protein per hour) was found in the mitochondrial fraction, especially the light mitochondrial fraction. A lower activity was found in the supernatant fluid. When Triton is omitted from the incubation mixture, there is less ammonia formation from BAA by the mitochondrial fraction (Table 3), thus indicating that the enzyme responsible for this reaction is partly in a bound form, from which it can be released by this surface active agent, as was also shown for

liver cathepsin by Gianetto and de Duve.[5] This finding would explain why BAA is not hydrolyzed appreciably by saline extracts of calf thymus.[3]

That the enzyme present in the lymph node mitochondrial fraction is similar to cathepsin B of beef spleen is supported by evidence presented in Tables 3 and 4. The lymph node enzyme is activated by cysteine and the

TABLE 3. EFFECT OF CYSTEINE, IODOACETAMIDE AND TRITON ON PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD BENZOYL-L-ARGININAMIDE

Complete system: 2.5 mg. of mitochondrial protein in 0.6 ml. of incubation mixture; pH 5.7; incubated for 180 min.; other additions as given in method section.

	NH_3 released per cent	*Specific activity*
Complete system	65	8.6
minus cysteine	11.7	1.6
plus 0.06 μmoles iodoacetamide	6.5	0.9
minus Triton	27.0	3.6

TABLE 4. pH DEPENDENCE OF THE PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD BENZOYL-L-ARGININAMIDE

Incubation volume, 0.6 ml.; "particles," 3.0 mg. protein; time, 180 min.

pH	*NH_3 released per cent*	*Specific activity*
3.3	12.1	1.3
4.2	31.4	3.3
5.0	50.0	5.1
5.7	81.0	9.0
6.5	10.0	1.1
7.3	3.6	0.4

activation is reversed by iodoacetamide, thus exhibiting a dependence on sulfhydryl groups similar to that of the spleen enzyme; the pH dependence of both the lymph node and spleen enzymes is also similar, with an optimum between pH 5 and 6.[6] Another activity of cathepsin B, that of transamidation, was tested in the presence of hydroxylamine as an acceptor. It is evident (Table 5) that the activity is dependent on hydroxylamine concentration, and that the extent of hydroxamic acid formation is much lower than the release of NH_3 from the peptide amide, similar to that described for cathepsin B of spleen.[4] The presence of cathepsin C, predominantly in the lymph node mitochondrial fraction, is indicated by the release of NH_3 from

glycyl-L-tyrosinamide. A similar localization of catheptic activity in liver was found by de Duve *et al.*,[1] who used hemoglobin as the substrate, and by Finkenstaedt,[8] who tested catheptic activity toward BAA and GTAA.

In the experiments with protein substrates, hemoglobin and serum albumin were used and their cleavage by the lymph node enzymes was ex-

TABLE 5. REACTION OF BENZOYL-L-ARGININAMIDE WITH HYDROXYLAMINE CATALYZED BY THE LYMPH NODE ENZYME PREPARATION

Mitochondrial fraction, 4 mg. protein per ml.; pH, 6.5; time, 90 minutes.

Hydroxylamine concentration	*Hydroxamic acid formed per ml.*	*NH_3 released per ml.*
M	*μmoles*	*μmoles*
0.37	0.43	
0.57	0.85	
0.80	1.16	15.0

TABLE 6. SUBCELLULAR DISTRIBUTION OF PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD SERUM ALBUMIN AND HEMOGLOBIN

pH 3.5.

Fraction	*Cathepsin units*	
	Albumin	*Hemoglobin*
Mitochondrial	0.40	3.65
Sup I	1.45	3.10
Microsomal	0	0
Sup II	1.45	3.40

amined. At pH 3.5, with hemoglobin as the substrate, proteolytic activity was found in both the mitochondrial and the supernatant fluid fractions. Serum albumin was predominantly cleaved by the supernatant fluid and no proteolytic activity towards either substrate could be demonstrated in the microsomal fraction (Table 6). Cleavage of an endogenous substrate was observed when the supernatant fluid was incubated for varying periods of time without the addition of albumin; during the first 30 minutes the endogenous substrate is preferred over the albumin and only during the subsequent period of incubation does the rate of cleavage of albumin exceed that of the endogenous substrate.

As shown in Table 7, the enzymic activity towards albumin is activated to some extent in the presence of cysteine and this activation is partly re-

versed by iodoacetamide. On the other hand, no effect of either cysteine or of iodoacetamide was observed on the enzymic activity toward hemoglobin.

The proteolytic activity towards serum albumin and hemoglobin has a wide pH range, as shown in Table 8. Hemoglobin was hydrolyzed to a

TABLE 7. EFFECT OF CYSTEINE AND IODOACETAMIDE ON PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD SERUM ALBUMIN AND HEMOGLOBIN
Sup I, 2.5 mg. protein; pH 3.5; complete system as described in methods section

	Cathepsin units	
	Albumin	*Hemoglobin*
Complete system	1.45	3.00
minus cysteine	0.75	2.70
plus 1.1 μmole iodoacetamide	1.00	2.75

TABLE 8. pH DEPENDENCE OF PROTEOLYTIC ACTIVITY IN LYMPH NODES TOWARD SERUM ALBUMIN AND HEMOGLOBIN

	Cathepsin units		
	Sup I		*Mit*
pH	*Albumin*	*Hemoglobin*	*Hemoglobin*
3.5	1.45	3.0	4.40
5.0	1.10	0	1.10
7.2	0.80	0.7	0

much greater extent at pH 3.5 than at pH 5.0, when the mitochondrial fraction was used as the source of enzyme; the supernatant fluid failed to hydrolyze this substrate at pH 5.0.

DISCUSSION

The present study has demonstrated that lymph nodes contain cathepsins which hydrolyze BAA and GTAA and which are similar to cathepsins B and C of spleen[6, 8, 15] and liver.[3] These enzymes were concentrated in the mitochondrial fraction, which also showed considerable hemoglobin splitting activity. Although it is not possible to decide whether the cleavage of hemoglobin is effected by the enzymes acting on BAA and GTAA, it seems

that cathepsins B and C are a part of the cathepsin complex of the mitochondrial fraction, which also causes hydrolysis of hemoglobin.[1]

Serum albumin was hydrolyzed to a greater extent by the supernatant fluid than by the mitochondrial fraction and the process was partly activated in the presence of cysteine, as also reported by Hess *et al.*[7] The partial dependence of the hydrolysis of albumin on the sulfhydryl groups might indicate the participation in this process of cathepsin B or C (or both) found in lymph nodes in the present study. It is reasonable to conclude that the hydrolysis of albumin and hemoglobin is a result of the cooperative action of several enzymes, which show different subcellular distribution and different dependence on SH groups.

The biological significance of the presence of these proteolytic enzymes in lymphatic tissue is of considerable interest. According to the data of Rothschild and Junqueira,[13] who determined catheptic activity in several rat organs, the highest activity (expressed as μg of tyrosine released from globin per 100 mg. of tissue) was found in the lymph nodes, followed by that of spleen, kidneys, and liver. The participation of these enzymes in the defense mechanisms of the lymphatic tissue against substances foreign to the body seems quite plausible. Proteolytic enzymes, active towards serum albumin and a variety of other proteins were described in polymorphonuclear leucocytes[9] and other phagocytic cells.[14]

The proteolytic enzymes found in the lymphatic tissue might be also implicated as participants in some pathological processes, such as the skin homograft reaction. The lytic breakdown of the graft occurs only after the appearance, in the vicinity of the graft, of a dense lymphoid infiltrate, considered to be a carrier of the immune antibodies. A release of the proteolytic enzymes from the lymphoid infiltrates in thyroids in Hashimoto's disease would affect the integrity of the thyroid follicles, resulting in a release of thyroglobulin into the circulation.

In view of the presence in the lymph nodes of proteolytic enzymes active towards serum albumin, the lymphatic tissue might be one of the sites of serum albumin catabolism. The active synthesis of immune proteins carried out by the components of that tissue could also partly derive its precursors from the products of local proteolysis.

SUMMARY

A study of the proteolytic activitity of subcellular fractions of rat lymph nodes has indicated the presence of cathepsins B and C, predominantly in the mitochondrial fraction. On the other hand, proteolytic activity toward serum albumin was found largely in the supernatant fluid obtained after

removal of the mitochondrial fraction, and strong activity toward hemoglobin was exhibited both by the mitochondrial and supernatant fluid fractions.

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EVENTS

AARON BROWN MEMORIAL LECTURE

Phi Delta Epsilon

21 March 1960

VARIATIONS ON AN ESOPHAGEAL THEME. Franz J. Ingelfinger, Professor of Medicine, Boston University School of Medicine.

Remarking at first that the esophagus was a rather humble organ to be deserving of the esoteric title of his lecture, Dr. Ingelfinger discussed various aspects of esophageal physiology and anatomy in a most humorous manner. Throughout, he illustrated the absurdity of over-simplification and of obstinacy in consideration of disease and physiological problems.

In addition to conveying material between the oropharynx and stomach and aiding actively in transport by peristalsis, the esophagus serves as a double barrier. Since the esophagus lies for the most part in the thoracic cavity, its pressure under usual conditions is subatmospheric (—5 cm. H_2O). Thus, a superior barrier must prevent influx of material from the pharynx, under atmospheric pressure, to the esophagus. An inferior barrier must similarly prevent reflux of material from the stomach, also under higher pressure, when a person is recumbent.

These barriers are explained in various ways by various clinicians, but the cogency of their arguments is reduced by a lack of experimental evidence. The superior barrier is most popularly considered as a blockage similar in action to a dam. The resting pressure in the region of this barrier (at the level of the cricoid cartilage) is significantly higher than that of the pharynx. On swallowing, the pressure rises in the pharynx, pushing food toward the barrier. A pressure drop occurs in the region of the barrier as the bolus approaches, the pressure returning to normal after the bolus has passed. According to the "dam" theory, dysphagia such as that found in bulbar poliomyelitis, might be the result of increased pressure from obstruction. However, the pressure changes are normal at the barrier; the abnormality lies in the absence of the initial pressure rise in the pharynx. Many doctors are so convinced of the "dam" theory that they are unwilling or unable to accept the concept implied—that is that the "blockage" results from an abnormality of *propulsive* power rather than from a narrowing of the esophageal lumen.

Of several theories advanced to explain the inferior barrier, Dr. Ingelfinger discussed two in detail. First, the diaphragmatic hiatus through which the esophagus enters the abdomen may function as a "pinchcock," especially in inspiration. This possibility has been examined by many investigators, and opinion is about equally divided as to whether the hiatus dilates or constricts as the diaphragm is pulled inferiorly. Its function must be only part-time at most. Second, an anatomical sphincter may be present in the esophagus. Pressure studies have indicated a region of high pressure similar to the superior barrier but no distinct anatomical region can be differentiated. The sphincter might thus be purely physiological, a nebulous concept.

A companion problem complicating the study of the inferior barrier is that of defining the contiguous boundaries of esophagus and stomach. A

morphological division based on muscle layers is impossible, since the muscle of the lower portion of the esophagus is continuous with that of the stomach; a division based on mucosal differences is unreliable since the gastric mucosa may extend for variable distances along the "esophagus." Furthermore, the pressure changes of the inferior sphincter are seen even when the gastric mucosa does extend significantly into the esophagus.

Dr. Ingelfinger turned to cardiospasm and constructed a clever travesty of superficial epidemiology. Cardiospasm involves the entire esophagus, causing diffuse motor paresis. It was considered of psychogenic origin, occurring particularly in persons with frustrated "oral aggressive impulses." Cardiospasm is, however, quite common in south-central Brazil, also the habitat of the cockroach-like insect *Triatoma megista*. The bite of this insect is known to lead, in time, to Chagas' disease, characterized especially by heart disease, parotid swelling, and symptoms of cardiospasm. Dr. Ingelfinger submits that this insect might be responsible for the incidence of cardiospasm in this geographic locale. In stressing that the etiology of this disease must be studied more thoroughly and that judgments on other diseases must not be made hastily, he admonished the audience to "beware of psychiatrists bringing etiologic gifts."

In conclusion Dr. Ingelfinger discussed esophageal motility. Despite differences in esophageal muscular composition, esophageal function throughout the animal kingdom is remarkably constant. For man, in whom the esophagus is one-third striated and two-thirds smooth muscle, and the dog, in which the esophagus is entirely striated muscle, there are similar esophageal control systems: the cholinergic nerves of Auerbach's plexus. Peristaltic waves move at similar rates. There is no evidence in man for reverse peristalsis as a mechanism or effect of reflux, although such reversal probably occurs in such a ruminant as the giraffe.

P. V. T.

BOOK REVIEWS

SEX DIFFERENTIATION AND DEVELOPMENT. Proceedings of a Symposium held at the Royal Society of Medicine, Wimpole Street, London, on 10 and 11 April 1958. C. R. Austin, Ed. Memoirs of the Society for Endocrinology, No. 7. Cambridge, University Press. x, 198 pp., illus. $8.50.

The latest memoir of the Society for Endocrinology presents the papers and discussions of a 1958 symposium on selected aspects of comparative and human sex morphology. It is gratifying that discussions of bacteria and invertebrate forms are included. Several authors examine the important question of genetic and hormonal controls of sex differentiation. The possibility of regulating the sex ratio at conception is considered, and so are the origin and grafting of ovarian germ cells and endocrine tissue, nuclear chromatin, failure of normal sexual differentiation, secondary manifestations of sexuality and other important topics. As is not unusual in a rapidly developing field, terminology occasionally presents a problem. (A separate conference essaying to standardize confused terms in the field of reproduction could be really valuable—conference organizers please take note!) Discussions of the papers, bibliographies, and an adequate index are included. Printing and binding are good.

Naturally no single symposium can include reviews of all aspects of such a broad topic. The papers which were presented, however, are authoritative, clear, and useful.

THOMAS R. FORBES

ENZYMES OF POLYNUCLEOTIDE METABOLISM. Jay S. Roth and others. *Annals of the New York Academy of Sciences,* vol. 81, art. 3, pp. 511-804. New York, 1959. $5.00.

This publication, the result of a conference held at the New York Academy in October 1958, is a collection of papers presented by specialists to specialists. Nonetheless, several of the papers admirably review the great progress made recently in the field of enzymes concerning polynucleotide metabolism and indicate some of the areas deserving of further study.

In Part I, the subject of discussion is crystalline pancreatic ribonuclease, one of the enzymes most thoroughly studied with respect to the relation of structure to function. The current position on the amino acid sequence of the enzyme, and other structural features, is given along with an excellent account of recent improvements in assay procedures for the enzyme. Some aspects of the relation of alterations in structure to immunochemical response and enzymatic activity are discussed.

Part II, Intracellular Ribonucleases, treats some of the difficult problems encountered in comparative studies on tissue homogenates and extracts. Distribution of RNases within the cell is discussed in conjunction with much valuable data on the apparent diversity of RNases in nature. Such studies assume immense importance since they should ultimately clarify the fundamental problems of hydrolytic enzyme action and intracellular function, after probable differences in primary, secondary, and tertiary structures of the enzyme protein are elucidated.

Part III of the monograph is concerned with the enzyme polynucleotide phosphorylase, the importance of which is stressed by Dr. Severo Ochoa in his introductory remarks on the macromolecular structure of RNA. Some new information is presented in the course of reviews on the mechanism of action of the enzyme, and of spleen and snake venom phosphodiesterases, two enzymes which have proved to be extremely useful in analytical work associated with investigations of polynucleotide structure. A few heuristic questions relating to the biosynthesis of RNA's are raised by E. S. Canellakis and in the words of Kai Linderstrøm-Lang, "Should we blame Prometheus because the mattress catches on fire?"

Part III is followed by a physico-chemically oriented section on the properties and structure of synthetic and other polynucleotides. In the course of this section, structural and functional questions about the relation of RNA to DNA are brought out and these lead to the final section on enzymes of polydeoxyribonucleotide metabolism. The current evidence on the mechanism of synthesis of DNA and polydeoxyribonucleotides, and on enzymes degrading DNA is discussed. The section includes a fine account of studies on the chemical synthesis of deoxyribo-oligonucleotides, and preliminary attempts to develop methods of end group analysis are described. Dr. Zamenhof in a brief article includes a bibliography of his work on the incorporation of analogues and biological assay for functional DNA, of interest in view of the work of Khorana and Kornberg.

This volume will not only serve the student as a useful reference but also offers a valuable survey of recent advances in this area of cellular physiology.

MARY E. KING

CARTER'S MICROBIOLOGY AND PATHOLOGY. By Alice Lorraine Smith. 7th ed. St. Louis, Missouri, The C. V. Mosby Company, 1960. 742 pp. $7.50.

This book is no more than it pretends to be, an introduction to microbiology and pathology for student nurses. The section on microbiology covers the field briefly and, unfortunately in some instances, even too briefly for nurses. The chapter on gram-positive cocci barely refers to the "hospital staphlococcus" problem, one in which the nurse plays as large a role as any member of the medical team.

The chapters dealing with physiology and biochemistry of microorganisms are so brief that little knowledge is gleaned from the reading. The author refers to "drug fastness," but offers no comments on the nature of this phenomenon or of any other aspects of the genetics of microorganisms. It is regrettable that there is no discussion of transformation or of the importance of DNA to the living organism.

The material on physical and chemical destruction of microorganisms is very good and is presented from the nurse's point of view. Even better is the section on pathology which might serve as an introduction to the subject, not only for student nurses but for students in medical technology. This material is clearly presented and well illustrated.

The bibliography consists primarily of references to textbooks, and although references to periodicals are included, they do not refer in many

instances to the most current publications. The excellent glossary in the back of this text will be useful to the student, as will the index which is well organized.

LEANOR D. HALEY

CANCER OF THE CERVIX, DIAGNOSIS OF EARLY FORMS. Ciba Foundation Study Group No. 3. G. E. W. Wolstenholme and Maeve O'Connor, Eds. Boston, Little, Brown and Company, 1959. 114 pp. $2.50.

Under the sponsorship of the Trustees of the Ciba Foundation, distinguished scientists from all over the world are invited to partake in small conferences dealing with controversial subjects in the field of medicine. The proceedings of such a study group are published in the booklet: *Cancer of Cervix, Diagnosis of Early Forms.*

The amount of research done in this field during the last two decades is vast, and the reader soon becomes aware that a less broad title would cover the seven papers dealing chiefly with the histopathology of early carcinoma of the uterus. All the authors are prominent scientists and they have studiously avoided over-interpreting their findings. The negative reports are in plurality, and discussions center mostly around suggested subclassifications of more classical clinical and pathological entities. Certain insight is obtained into how borderline cases of carcinoma-*in-situ* are taken care of in various centers of Europe and the United States.

Among the positive results is a report on the morphological changes that occur in the cervix uteri under the influence of ovarian hormones. The possibility of carcinoma-*in-situ* being withdrawn into the endocervical canal during the postpartem period is discussed. In a similar way, many endocervical carcinoma begin in the epithelium covering the indrawn cervical glands of the post-menopausal patient.

As a whole, the booklet is of value as a supplement to more comprehensive works on the subject.

STANLEY ZERNE

CONVOCATION

Commemorating The Sesquicentennial Of The Founding Of The Medical Institution Of Yale College

Program

Friday, October 28th

Morning Session, Ten-thirty o'clock, Sprague Hall

PRESIDING— Vernon W. Lippard, Dean, School of Medicine

WELCOME— A. Whitney Griswold, President of the University
Abraham A. Ribicoff, Governor of the State of Connecticut

GREETINGS—John N. Gallivan, Representing the Connecticut State Medical Society
Norman A. Welch, Representing the American Medical Association
John McK. Mitchell, Representing the Association of American Medical Colleges

ADDRESS— "Medical Science in the Twentieth Century"
Sir Howard Florey, Professor of Pathology, University of Oxford

Afternoon Session, Two o'clock, Sprague Hall

PRESIDING— George W. Pierson, Larned Professor of History

"Medical Education at Yale in the Twentieth Century"
Vernon W. Lippard, Dean

"Medical Research at Yale in the Twentieth Century"
Averill A. Liebow, John Slade Ely Professor of Pathology

"Medical Science and the Future"
C. N. H. Long, Sterling Professor of Physiology

"Clinical Medicine and the Future"
Paul B. Beeson, Ensign Professor of Medicine

Reception, Four-thirty o'clock, Sterling Hall of Medicine

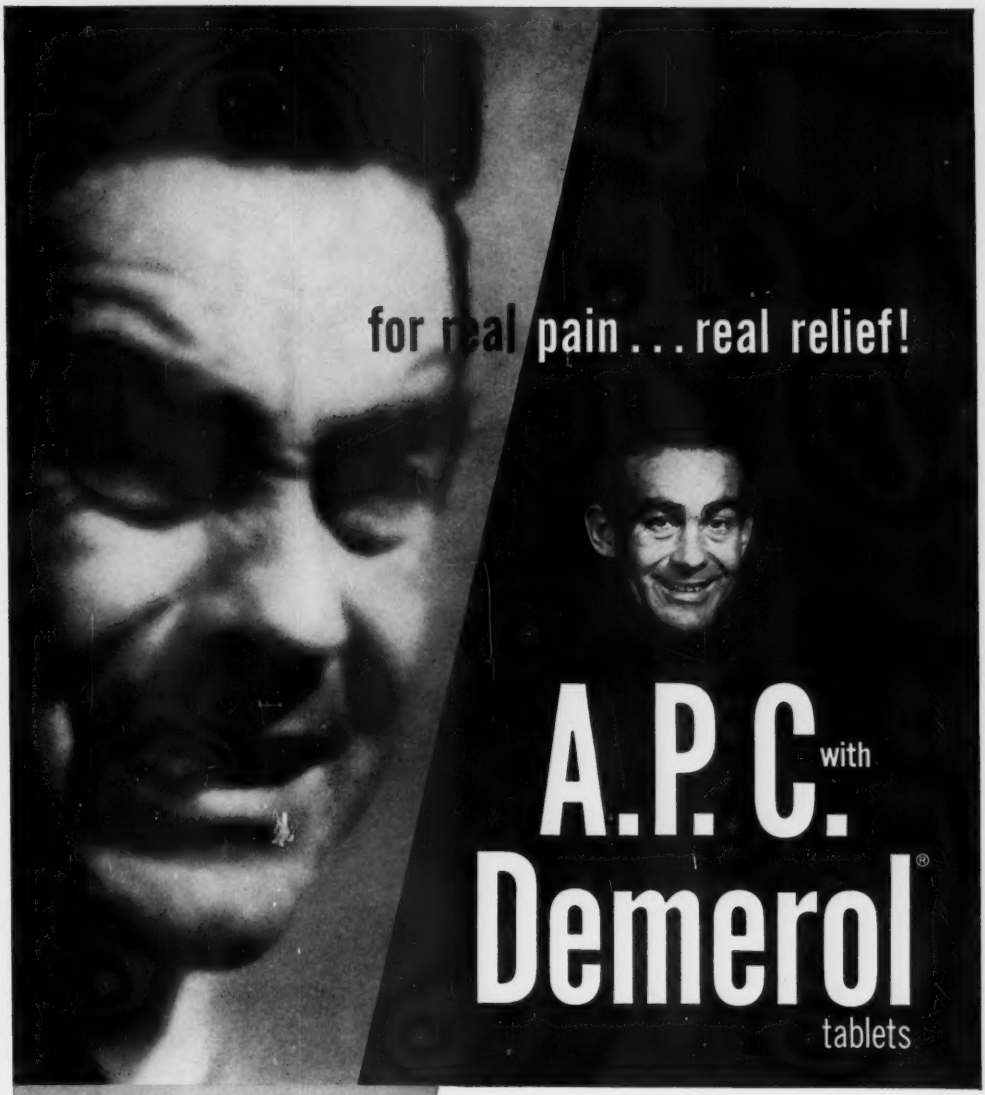
Saturday, October 29th

Morning Session, Nine-thirty o'clock, Yale Medical Library

PRESIDING— Frederick G. Kilgour, Librarian, School of Medicine

"Medicine in the Nineteenth Century"
Lloyd G. Stevenson, Dean, Faculty of Medicine, McGill University

"The Medical Institution of Yale College in the Nineteenth Century"
Whitfield J. Bell, Jr., Research Associate in History



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